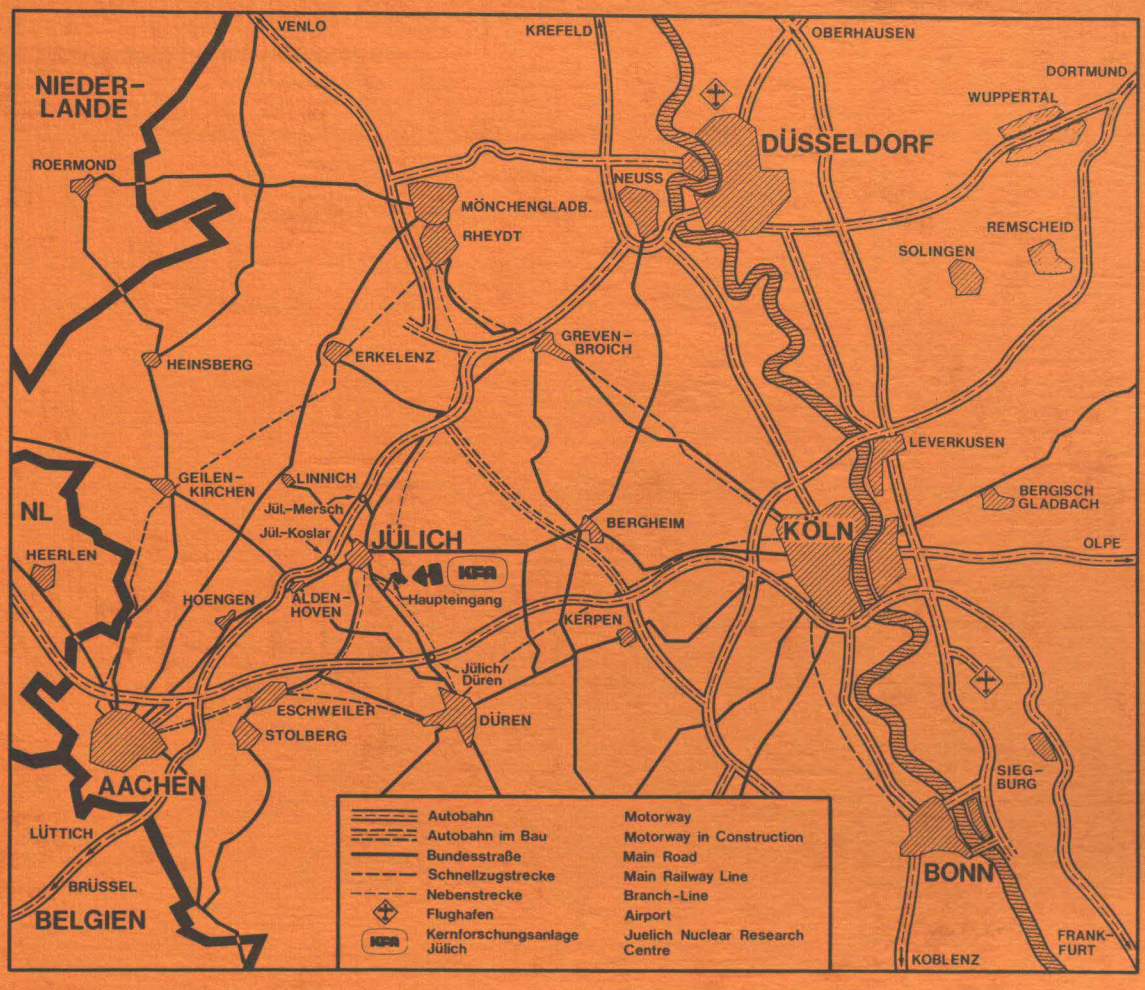
KERNFORSCHUNGSANLAGE JÜLICH GmbH
Zentralabteilung Chemische Analysen

Sampling and Sample Preparation Methods for the Analysis of Trace Elements in Biological Material

by

B. Sansoni and V. Iyengar

Jül-Spez-13
Mai-1978
ISSN 0343-7639



Als Manuskript gedruckt

Spezielle Berichte der Kernforschungsanlage Jülich - Nr. 13

Zentralabteilung Chemische Analysen Jül - Spez - 13

Zu beziehen durch: ZENTRALBIBLIOTHEK der Kernforschungsanlage Jülich GmbH,
Jülich, Bundesrepublik Deutschland

Sampling and Sample Preparation Methods for the Analysis of Trace Elements in Biological Material

by

B. Sansoni and V. Iyengar
with a contribution by V.K. Panday

Invited Review Paper International Symposium on
Nuclear Activation Techniques in the Life Sciences,
International Atomic Energy Agency
Vienna, 22 to 26 March 1978

CONTENT

PREFACE/VORWORT 7

ABSTRACT/ZUSAMMENFASSUNG 9

1. PRINCIPLE OF TRACE ELEMENT ANALYSIS 13

1.1 The analytical problem 13
1.2 Trace analysis procedures 13
1.3 General steps of trace element analysis 14
1.4 Presentation of trace analysis data 14
1.5 Trace elements analysed in biomedical samples 14
1.6 Classification of biomedical samples according to similar major and minor element composition 15
1.7 Literature 19

2. SAMPLING OF BIOLOGICAL MATERIAL 21

2.1 Fundamental aspects 21

2.1.1 Basic considerations 21
General reviews 21
2.1.2 Fundamental laws 21
Representative mean composition 21
Representative variance 21
Accuracy 21
Efficiency 21
2.1.3 General sampling methods 21
2.1.4 Sampling units 22
2.1.5 Influence of various parameters on the optimal sample size 22
2.1.5.1 Sample size of particulate matter 22
2.1.5.2 Constant number of particles per sample 22
2.1.5.3 Sample size and number of particles 22

2.2 Basic hazards 22

2.2.1 Contamination by elements 22
2.2.2 Loss of trace elements 23
2.2.3 Change in mean composition 23

2.3 Special restrictions 23

2.3.1 Samples of human origin (in vivo samples) 23
2.3.1.1 Stringent medical preparations 23
2.3.1.2 Legal regulations for autopsy (in vitro samples) 27
2.3.1.3 Without stringent medical precautions 27
2.3.1.4 Physiological and pathological influences 27
Time dependence 27
Pre- and post natal state 27
Age dependency 27
2.3.2 Samples of animal origin 27
2.3.3 Other biological samples 27

2.4 Outlining the sampling program 27
2.5 Solid biomedical samples 27
2.6 Sampling procedures for solids 29
2.7 Liquid biomedical samples 29

2.8 Sampling procedures for liquids 29
2.9 Preliminary preservation 34
2.10 Transport to the laboratory 34
2.11 Removal of interfering components 34
2.11.1 Solid samples 34
2.11.2 Liquid samples 34
2.12 Final preservation 34
2.13 Storage 35
2.13.1 Purpose 35
2.13.2 General considerations 35
2.13.3 Packing 35
2.13.4 Storage in preservative solution 35
2.13.5 Container material 35
2.13.6 Freezing 35
2.13.7 Drying 36
2.13.8 Storage of solids 36
2.14 Literature 37

3. SAMPLE PREPARATION OF BIOLOGICAL MATERIAL FOR TRACE ELEMENT ANALYSIS 41
3.1 General considerations 41
3.1.1 Principle 41
3.1.2 Requirements 41
3.1.3 Shortcomings 43
3.1.4 Reviews and selected references 43
3.2 Basic hazard 43
3.2.1 Contamination by elements 43
3.2.2 Loss of trace elements 45
3.2.3 Change in mean composition 45
3.3 Outlining the sample preparation plan 46
3.4 Sample preparation of solid samples 46
3.4.1 Particle size reduction 46
3.4.1.1 Methods for hard and semi hard tissues 46
3.4.1.2 Soft tissues 48
3.4.1.3 Drying before particle size reduction 48
3.4.2 Homogenisation 48
3.4.3 Homogeneity tests 49
3.4.4 Subsampling 49
3.4.5 Preparation of solid samples for instrumental measurement 49
3.5 Liquid samples 51
3.5.1 Homogenisation 51
3.5.2 Phase separation 51
3.5.3 Preconcentration of liquid phase 51
3.5.4 Solid phase 54
3.5.5 Homogenisation of the liquid 54
3.5.6 Homogeneity test for the liquid 54
3.5.7 Subsampling 54
3.5.8 Preparation of liquid samples for instrumental methods 54

3.6 Preservation and final storage 54

3.7 Sample preparation methods and some special considerations 54

Sample preparation for neutron activation analysis 54
Sample preparation for atomic absorption spectrometry 54
3.7.1 Hard tissues 55
3.7.2 Semi hard tissues 55
3.7.3 Soft tissues 56
Autopsy samples 56
Biopsy samples 57
3.7.3.1 Liver 57
3.7.3.2 Kidney 57
3.7.3.3 Brain and lung 57
3.7.3.4 Placenta 57
3.7.4 Biological fluids 57
3.7.4.1 Blood 58
3.7.4.2 Serum 58
3.7.4.3 Plasma 58
3.7.4.4 Erythrocytes 58
3.7.4.5 Urine 58
3.7.4.6 Milk 59
3.7.4.7 Sweat 59
3.7.4.8 Excreta 59

3.8 Literature 60

4. ASHING 65

4.1 Introduction 65

4.2 Dry ashing 65

4.2.1 Dry ashing in muffle furnace 67
4.2.2 Combustion in oxygen flask 67
4.2.3 Combustion in oxygen bomb 70
4.2.4 Combustion in gas stream 70
4.2.5 Combustion in oxygen/hydrogen flame 70
4.2.6 Pyrolytic decomposition 70
4.2.7 Low temperature ashing in oxygen plasma 70

4.3 Wet ashing 71

4.3.1 Higher temperature, normal pressure 71
4.3.2 Wet ashing in closed system under pressure 72
4.3.3 Low temperature wet ashing with ·OH radicals from Fenton's reagent H_2O_2/Fe^{2+} 72

4.4 Other methods 73

4.4.1 Oxidative fusion 73
4.4.2 Oxidation in nitric acid vapour 73
4.4.3 Oxidation with ozone 73
4.4.4 Reductive decomposition 74
4.4.5 Decomposition with halogens 74
4.4.6 Enzymatic/hydrolytic decomposition 74

4.5 Literature 75

5. CONCLUSIONS 79

PREFACE

The following report was presented in a short form as an opening lecture at the International Symposium on Nuclear Activation Techniques in the Life Sciences, International Atomic Energy Agency, Vienna, 22-26 May 1978.

It is based on earlier talks on sampling, sample preparation and ashing of environmental and biological samples by one of the authors (S.) during the period 1965-77 and special experience of the other author (I.) in activation analysis of human samples. Therefore, the biological material considered here deal mainly with human samples. Both the authors also worked at the Institute of Radiation and Environmental Research at Neuherberg near Munich and at the Nuclear Research Establishment, Jülich.

The following chapters are an attempt to systematically treat the subject of sampling and sample preparation of biological material with reference to trace element analysis. A survey of the available ashing methods to be used for such samples has also been made. During the preparation of this review, it was obvious to the authors, that it is difficult, to establish a standard scheme of sampling and sample preparation valid for all cases, like in classical qualitative analysis of cations. Moreover, because of the lack of standardized procedures for sampling, sample preparation and ashing operations in case of biological samples, individual variations and modifications may be made by different laboratories according to their own knowledge and experience. Nevertheless, a systematic approach is still useful and may help not only during investigation of the analytical procedure, but also in establishing the sampling and sample preparation plan before beginning the analysis. As pointed out in a plenary lecture (S.) on the principles of trace element analysis and its future trends at the IUPAC-Postcongress Symposium on „Future Trends in Chemistry", Kyoto, 1977, the sampling, sample preparation and data evaluation are aspects of trace analysis, where future investigations and progress will not only be important but necessary.

The authors will gratefully acknowledge comments, further informations and suggestions on this review, since it is planned to condense the content to a sampling and sample preparation chart for trace element analysis of biological material.

The authors are grateful to Dr. R.K. Iyer and Dr. V.K. Panday, guest scientists from BARC, India, at the Central Department of Chemical Analysis KFA, for suggestions during the preparation of this manuscript; to Dr. W. Manz, Director of the Central Library of KFA and his coworkers for acquiring the literature; to Miss S. Buchholz and Mr. K. Prendergast for help in preparing the tables; to Mrs. A. Brunner for typing and Mr. W. Arras, Mr. H.P. Pelzer, Mr. T. Rüttgers from the printing section of KFA for printing the manuscript. One of the authors (S.) likes to further thank Prof.Dr. R. Bock and Ing. W. Kracke for valuable discussions on ashing methods some time ago. The other author (I.) is thankful to Dr. K. Kasperek, Institute of Medicine, KFA (Director: Prof.Dr. L.E. Feinendegen) for many valuable discussions.

Jülich, May 1978

B. Sansoni, V. Iyengar

VORWORT

Der folgende Bericht wurde in verkürzter Form als Eröffnungsvortrag auf dem International Symposium on Nuclear Activation Techniques in the Life Sciences, Internationale Atomenergiebehörde, Wien, 22. bis 26. Mai 1978, gehalten.

Er ist aus früheren Vorlesungen und Vorträgen des einen Autors (S) über Probenahme, Probenvorbereitung und Veraschung von Umwelt- und biologischem Material während der Jahre 1965 bis 77 und den besonderen Erfahrungen des anderen Autors (I.) mit der Aktivierungsanalyse medizinischer Proben hervorgegangen. Daher umfaßt das hier berücksichtigte biologische Material vorwiegend Proben aus der Humanmedizin. Beide Autoren arbeiteten sowohl in der Gesellschaft für Strahlen- und Umweltforschung GmbH München in Neuherberg, als auch in der Kernforschungsanlage Jülich GmbH in Jülich.

In den folgenden Kapiteln wird versucht, die Probenahme und Probenvorbereitung von biologischem Material vom Standpunkt der Spurenelementanalyse systematisch zu behandeln. Ein Überblick über die geläufigsten Veraschungsmethoden schließt sich an. Während der Ausarbeitung dieses Berichtes wurde es den Autoren klar, wie schwierig es ist, ein allgemeines und umfassendes Schema der Probenahme und Probenvorbereitung, vergleichbar etwa dem klassischen Trennungsgang der Kationen, aufzustellen. Außerdem modifizieren die verschiedenen Laboratorien die Arbeitsweisen nach ihren eigenen Erfahrungen und Gepflogenheiten, da es für Probenahme und Probenvorbereitung von biologischem Material noch kaum Standardverfahren gibt. Es ist die Hoffnung der Autoren, daß die vorliegende systematische Zusammenstellung trotzdem nützlich ist und nicht nur bei der Ausarbeitung der Analysenvorschrift, sondern auch bei der Aufstellung des Probenahme- und Probenvorbereitungsplanes vor Beginn der eigentlichen Analyse eine Hilfe sein kann.

Wie der eine Autor (S.) kürzlich in einem Vortrag über die Grundlagen der Spurenelementanalyse und deren zukünftiger Entwicklung auf dem IAEA-Nachkongreß-symposium über „Zukünftige Entwicklungen der Chemie", 1977 in Kyoto, betont hat, gehören Probenahme, Probenvorbereitung und Datenbeurteilung zu den derzeit wichtigsten Teilschritten der Spurenelementanalyse, auf denen noch viel zukünftige Entwicklung notwendig sein wird.

Die Autoren sind dankbar für Hinweise, weitere Informationen und Vorschläge. Der Inhalt vorliegender Kapitel über Probenahme und Probenvorbereitung soll in einer Wandkarte zusammengefaßt werden, die im Laboratorium gewissermaßen als Checkliste für die Spurenanalyse von biologischem Material dienen kann.

Die Autoren danken besonders den Herren Dr. R.K. Iyer, Leiter der Sektion Aktivierungsanalyse und Dr. V.K. Panday, Leiter einer Gruppe Atomabsorptions-Spektralanalyse im BARC, Indien, beide zur Zeit Gastwissenschaftler in der Zentralabteilung für Chemische Analysen der ZCH, für Hinweise und Korrekturlesen während der Herstellung des Manuskriptes. Der Direktor der Zentralbibliothek der KFA, Dr. W. Manz und seine Mitarbeiter halfen bei der Literatursuche und -beschaffung, Frl. S. Buchholz und Herr stud. med. K. Prendergast bei der Herstellung verschiedener Tabellen. Frau A. Brunner danken wir für das mehrfache Schreiben des Manuskriptes und den Graphischen Betrieben der KFA, insbesondere deren Leiter, Herrn W. Arras sowie den Herren H.P. Pelzer und T. Rüttgers für den Druck. Einer der Autoren (S.) dankt Herrn Prof. Dr. R. Bock und Ing. W. Kracke für Diskussionen über die Veraschung, der andere (I.) Herrn Dr. K. Kasperek vom Institut für Medizin der KFA (Direktor: Prof. Dr. L.E. Feinendegen) für zahlreiche wertvolle Diskussionen.

Jülich, im Mai 1978

B. Sansoni, V. Iyengar

ABSTRACT

In general, trace element analysis of biological material includes the following steps: Formulation of the question to be answered by the analytical data, sampling, sample preparation, ashing, dissolution, preconcentration, separation, preparation of the measurement sample, physical measurement (integral, spectral, calibration), data processing, data evaluation and answering the question. Both sampling and the sample preparation are crucial stages for any trace analysis. Knowledge of numerous factors and strict adherance to all the requirements is unavoidable, if results are to be reliable.

Sampling of biological material involves some fundamental aspects such as representative mean composition, representative variance, accuracy and efficiency of sampling. The general sampling methods used may be random, systematic or of differentiated types. Various parameters such as size of the totality of material, number and size of the particles, distribution of trace elements between different types of particles influence the optimal size of the sample.

There are some basic hazards in trace element analysis, which arise from contamination of the sample by the elements, loss of trace elements and change in mean composition with respect to the elements to be analysed. Contamination of the sample may occur from the environment, the sampling operation itself and the operating personnel. Trace elements may be lost by volatilization during drying and ashing, adsorption on container walls and handling tools, overlooked differences in the chemical state of the element and also by sputtering and spraying in case of liquids. The changes in the mean trace element composition of the sample may result from the physical or chemical changes and by redistribution of the elements.

The main sampling operations for solid tissues include the selection of an optimal sampling method, the sampling site and the preparation of the sampling equipment. Liquid samples require additional treatment such as the use of a stabilizer and phase separation. It is also necessary to obtain timed collection for samples such as urine and feces. Special restrictions have to be observed for both liquid and solid samples due to medical reasons, especially for in-vivo samples. Some sort of a preliminary preservation of both the solid and liquid samples is necessary during transport to the laboratory.

Sample preparation of biological material is necessary in order to prepare the samples for obtaining the analytical signal. A few requirements such as a well defined analytical programme, prior knowledge of the approximate elemental composition of the sample matrix and a reasonably clean bench to suit the low concentration level analytical work and adequate precautions against the basic hazards mentioned above are some of the basic necessities.

For solid samples, the sample preparation steps include particle size reduction by fragmentation, drying and grinding, followed by homogenisation and homogeneity test, preconcentration for the instrumental measurement and subsampling. For liquid samples, which are generally suspensions or emulsions, the following steps are necessary: Homogenisation, phase separation, preconcentration by evaporation, drying, distillation, ashing, protein precipitation, subsampling and preconcentration for instrumental measurement. Final preservation and storage are necessary if further analysis is not taken up immediately. Storing the dried samples at 2-4 °C and gamma ray irradiation reduce the speed of microbial and chemical decay. Exclusion of air dust and humidity is mandatory.

Ashing allows to eliminate the large excess of organic matrix within the biological sample by converting it into suitable gaseous compounds and their subsequent volatilisation, in order to analyse the trace elements in the inorganic residue without interferences. For pure instrumental analysis, ashing is an additional tool for preconcentration with factors of about hundred for fresh soft tissue, a factor of about four for freeze dried soft tissues and a factor of five to thirty for freeze dried body fluids. The chemical principle is mainly a powerful oxidation to form volatile CO_2, H_2O, HCl, SO_3 or N_2. Only in few cases, reduction to H_2O, H_2S, PH_3, NH_3, HF, HCl, HB, HI, As, Hg, Zn, Cd etc. is used. Ashing should remove all organic compounds of the sample completely, retain the traces of elements to be determined quantitatively within the residue without contamination by these elements from outside.

Ashing methods can be classified (a) depending on the application of a gaseous or liquid reagent into dry or wet ashing, (b) according to the temperature applied into higher and lower temperature and (c) according to the pressure into high or normal pressure ashing methods.

Among dry ashing methods, oxidation with air in a muffle furnace at 400 to 800 °C, combustion in an oxygen flask, oxygen bomb, oxygen gas stream, oxy-hydrogen flame, pyrolytic decomposition under inert or hydrogen gas and lower temperature ashing in oxygen gas plasma at low pressures and temperatures below 150 °C are discussed.

Wet ashing is mainly based on oxidation by concentrated strong oxidizing mineral acids such as HNO_3, H_2SO_4, $HClO_4$, (HF), hydrogen peroxide and their mixtures. They can be used either under normal pressure or under high pressure in a closed, teflon coated bomb. Relatively new among wet digestion methods is wet ashing by ·OH radicals from Fenton's reagent H_2O_2/Fe^{2+} under normal pressure at temperatures below 110 °C in an open or a closed system.

Other ashing techniques, used only occasionally or described in the last few years, such as oxidative fusion with nitrates, oxidation with nitric acid vapour, ozone, reductive decomposition, decomposition with halogens, enzymatic or hydrolytic decomposition are also covered briefly.

For the most frequently applied lower temperature dry ashing with oxygen gas plasma, wet ashing with $HNO_3/HClO_4$, low temperature wet ashing with radicals from H_2O_2/Fe^{2+}, the combustion in an oxy-hydrogen flame ard dry ashing in the muffle furnace, more details are given.

In conclusion, sampling and sample preparation of biological material need further investigation and strict implementation of the various procedural details. More details concerning sampling and sample handling procedures should be mentioned while reporting analytical procedures and results in order to help judging the reliability of data. From data evaluation point of view for improving the reliability and especially the accuracy of biological trace element data, establishing standardized procedures also for sampling and sample preparation in addition to other steps of trace analysis seems to be inescapable for the future.

ZUSAMMENFASSUNG

Im allgemeinen besteht die Spurenanalyse von Elementen in biologischem Material aus der Formulierung einer durch das Analysenergebnis zu beantwortenden Frage, der Probenahme, Probenvorbereitung, Veraschung, Auflösung, Vorkonzentrierung, Trennung, Vorbereitung des Meßpräparates, physikalischen Messung (integral, spektral, Kalibrierung), Datenverarbeitung, Datenbeurteilung und schließlich Beantwortung der eingangs gestellten Frage. Dabei sind Probenahme und Probenvorbereitung besonders kritische Schritte. Um zuverlässige Analysenergebnisse zu erhalten, müssen zahlreiche Einzelheiten genau bekannt sein und sorgfältig eingehalten werden.

Das **biologische Material** des menschlichen Organismus läßt sich einteilen in harte, halbweiche und weiche Festproben sowie Körperflüssigkeiten, wobei mehr als hundert verschiedene Probenarten zu unterscheiden sind. Das Gerüst des Körpers bilden die Elemente C, H, N, O, P, S, bei harten Proben auch Ca. Hauptbestandteile der Körperflüssigkeiten sind Ca, Cl, K, Mg, Na. Einzelne wenige Probenarten sind durch die Nebenbestandteile Al, Ba, Cl, Cu, F, Fe, I, K. Mg, Na, Si, Sn, Si oder Zn charakterisiert. Als essentielle, vermutlich oder nicht essentielle, toxische und sonstige Elementspuren wurden darüber hinaus bisher unter verschiedenen Bedingungen im menschlichen Körper noch Ag, As, Au, B, Ba, Be, Bi, Br, Bd, Ce, Co, Cr, Cs, Cu, F, Fe, Ga, Ge, Hg, I, Li, Mn, Mo, Nb, Ni, Pb, Po, Rb, Sb, Sc, Se, Te, Th, Ti, U, V, W, Zr untersucht. Das sind zusammen etwa 60 Elemente.

Eine Klassifizierung biomedizinischer Materialien in Gruppen mit gleichen höchsten Elementgehalten, ergab daß die höchsten Gehalte an Al in Lunge, Lymphknoten und Nägeln; Ca in Knochen und Zahn; Cl in Gehirn, Faeces, Niere; F in Knochen und Zahn; Fe in Leber, Milz, Placenta, Erythrozyten; I in Schilddrüse; K in Faeces, Herz, Muskel, Zunge, Gallensaft; Mg in Knochen, Zahn, Faeces, Gallensaft, Schweiß, Urin; P in Knochen, Gehirn, Faeces, Leber, Milz Zahn; S in Haar; Si in Lymphknoten; Zn in Prostataflüssigkeit vorhanden sind.

Die **Probenahme** von biologischem Material beruht vor allem auf einer repräsentativen mittleren Zusammensetzung sowie repräsentativen Varianz der Probe im Hinblick auf die zu beurteilende Gesamtheit an Material und die zu bestimmenden Elemente. Außerdem sind Richtigkeit und Wirtschaftlichkeit zu berücksichtigen. Die Probenahme kann zufällig, systematissh oder differenziert vorgenommen werden. Die optimale Probengröße hängt unter anderem ab vom Umfang der zu beurteilenden Gesamtheit, der Anzahl und Größe der Teilchen sowie Verteilung der Elementspuren zwischen verschiedenartigen Teilchen ab.

Drei allgemeinen **Gefahren** sieht sich der Spurenanalytiker auch bei der Probenahme und Probenvorbereitung gegenüber, der Einschleppung von Verunreinigungen, dem Verlust von Elementspuren und Änderungen der mittleren Zusammensetzung der Probe im Hinblick auf die zu bestimmenden Elemente. Die Verunreinigung kann aus der Umgebung, von der Probenahme selbst sowie vom Analytiker stammen. Spurenelemente können durch Verdampfen während des Trocknens und Veraschens, durch Adsorption an Gefäßwänden und Geräten, durch übersehene Unterschiede der chemischen Form des Elementes und darüber hinaus durch Versprühen und Verspritzen von Lösungen verloren gehen. Änderungen der mittleren Elementspurenzusammensetzung der Probe können von physikalischen und chemischen Veränderungen der Probe und einer dadurch bedingten Umverteilung der Elementspuren stammen.

Die Probenahme **fester** Proben beinhaltet die Auswahl und Festlegung der geeignetsten Probenahmemethode, des Ortes der Probenahme und der Vorbereitung des Probenahmegerätes. **Flüssige** Materialien erfordern außerdem oft die Zugabe von Stabilisatoren sowie Phasentrennungen. Proben wie Urin und Faeces müssen häufig zu bestimmten Zeiten gesammelt werden. Medizinische Einschränkungen sind insbesondere bei in vivo Proben zu berücksichtigen. Der Transport in das Laboratorium erfordert häufig eine kurzfristige Konservierung von biologischem Material.

Durch die **Probenvorbereitung** soll das biolgoische Material in eine Form gebracht werden, welche die nachfolgenden Analysenschritte bis zur Messung des Analysensignals erlaubt. Voraussetzung dafür ist unter anderem ein gut ausgearbeiteter Analysenplan, vorherige Kenntnis der ungefähren Zusammensetzung der Probenmatrix, geeignete Arbeitsbedingungen für das Arbeiten mit niedrigen Spurengehalten sowie ausreichende Vorkehrungen, um die eingangs genannten drei Hauptgefahren des spurenanalytischen Arbeitens möglichst gering zu halten.

Bei **Festproben** beinhaltet die Probenvorbereitung im allgemeinen eine Verkleinerung der Teilchengröße durch Grobzerkleinerung, Trocknen und Mahlen mit anschließender Homogenisierung mit Homogenitätstests, Anreicherung für die rein instrumentelle Bestimmung sowie eine Probenverkleinerung, gegebenenfalls unter Wiederholung vorausgegangener Teilschritte. **Flüssige** Proben, die fast ausschließlich als Suspensionen oder Emulsionen vorliegen, erfordern meist entweder eine Homogenisierung oder eine Phasentrennung, gefolgt von der Anreicherung durch Eindamp-

fen, Trocknen, Destillation, Veraschung, Proteinfällung, Probenverkleinerung und Anreicherung bei instrumenteller Bestimmung. Bei Verzögerung der anschließenden Analyse sind eine Konservierung und Aufbewahrung erforderlich. Durch die Aufbewahrung trockener Proben bei 2 bis 4°C sowie durch Gammabestrahlung verringert sich die Geschwindigkeit des mikrobiellen und chemischen Abbaues. Der Ausschluß von Luftstaub und Luftfeuchtigkeit während der Arbeiten ist notwendig.

Veraschungsmethoden werden nach der (a) Anwendung gasförmiger oder flüssiger Reagenzien in Trokken- und Naßveraschung, nach der (b) Arbeitstemperatur in Höher- und Niedrigertemperatur-Veraschung sowie nach dem (c) angewendeten Druck in Hoch- und Normaldruck-Verfahren unterschieden.

Bekannte Beispiele für **Trockenveraschungen** sind die Oxydation mit Luft im Muffelofen, die Verbrennung in der Sauerstoffflasche, Sauerstoffbombe, im Sauerstoffstrom und in der Knallgasflamme, die pyrolytische Zersetzung unter Inertgas oder Wasserstoff sowie die Veraschung im Sauerstoffplasma unter niedrigem Druck bei Temperaturen unter 150°C.

Die **Naß**veraschung beruht meist auf einer kräftigen Oxydation mit konzentrierten Mineralsäuren, die oxydierend und teilweise zusätzlich dehydrierend wirken. Beispiele sind HNO_3, H_2SO_4, $HClO_4$, (HF) und deren Gemische. Hinzu kommt noch H_2O_2. Die Oxydation erfolgt entweder unter normalem Druck im offenen Quartzglassystem oder unter hohem Druck in der mit Teflon ausgekleideten Bombe. Jüngeren Datums und erst wenig bekannt ist die schonende Naßveraschung großer Probenmengen bis in den Centi- und Kilogrammmaßstab durch die mit katalytischen Mengen Eisen(II) aus H_2O_2 erzeugten Radikale unter normalem Druck bei Temperaturen unter 110°C im offenen oder geschlossenen System. Die am häufigsten verwendeten Methoden der Trockenveraschung im Muffelofen sowie im Sauerstoffplasma, die Verbrennung in der Knallgasflamme, die Naßveraschung mit HNO_3/H_2SO_4 sowie mit H_2O_2/Fe^{3+} werden etwas ausführlicher behandelt.

Einzelne andere Veraschungstechniken, die entweder nur gelegentlich verwendet werden oder erst kürzlich beschrieben wurden, sind die Nitratschmelze, Gasphasenoxydation mit HNO_3 oder Ozon, die reduktive Zersetzung, die Reaktion mit Halogen und gelegentlich auch die enzymatische, hydrolytische sowie strahlenchemische Zersetzung.

1. PRINCIPLE OF TRACE ELEMENT ANALYSIS

A few years ago, H.J.M. Bowen in England (3) has estimated, that all over the world about six billion single element analyses in biological material are carried out per year. If we take out major and minor elements such as Ca, Cl, K, Mg, N, Na, P, S, there still remain about sixteen million determinations per year. The Advisory Group to this Symposium in 1976 has discussed, that more than half of this number of trace analysis data is wrong or not reliable in the sense, that the medical and legal conclusions drawn from them are wrong or not reliable. The physician, epidemiologist, ecologist, administrator or lawyer usually is shocked by these facts. The analytical chemist however, will not be surprised. He knows, that trace analysis of any material in the ppm-level and below, in contrast with other disciplines of modern science, is a mixture of science and art, in which the principles are quite clear and well understood, but details are more important than the principles (1–13, 17–25).

1.1 The analytical problem

Trace element analysis compared with major or minor constituent analysis is characterised by the extremely small absolute amounts of elements to be determined and the enormous, often million to billion fold excess of matrix. This may seriously inferfere with the quantitative determination of the trace constituent, apart from the interferences from other constituents. Thus, in biological samples the organic matrix in most cases has to be removed before starting the determination. For biomedical samples, additional problems are the preservation of unstable biomatrix before starting the laboratory work and medical or legal restrictions for sampling parts of a living or dead human organism by biopsy or autopsy.

Let us give an example for the extremely small amounts and contents in the ppb range. 1 ppb of an element for 1 gram of sample corresponds to an absolute amount of only 1 nanogram. Analysis of 1 ppb of the element in the biomatrix corresponds to the finding of four people within all four billion individuals living on the earth, when observing them by a binocular from a satellite hundred kilometres away. The four single people alone on a barren plane would correspond to a pure aqueous solution without interferences. Analysis of this 1 ppb within an interfering matrix, however, would be comparable to the identification and counting of the four single people within the whole mankind, concentrated on an area of about 2000 square kilometers, 50x40 km respectively, if two persons are standing in an area of one square meter.

1.2 Trace analysis procedures

The qualitative characterisation as well as the quantitative determination of an element in a given material is primarily based on the measurement of a characteristic signal produced by this element, either by its atomic nuclei, electron shells, molecules or even phases or surfaces. Frequently, the energy level of this signal is characteristic of the nature of the element and its intensity of the amount of element. Knowledge of the mass of the sample gives the content or relative amount.

Unfortunately, there are no absolute analytical signals. Therefore, the mathematical relationship (calibration curve) between the intensity of the signal and the amount of element must be known. In the simplest case, one is proportional to the other. In practice, however, due to interference from other constituents of the sample, and from other sources, the function becomes much more complicated. Nine different types of calibration methods have been discussed by H. Kaiser (5).

The process of calibration requires comparison with analytical signals from known amounts of the element to be determined. In most cases calibration has to be obtained with known amounts of the element present in the same or a comparable matrix. Thus standard samples for a large variety of material become necessary.

When the content of the element in the sample is too small, this gives rise to a weak analytical signal which cannot be discerned from the background signal. In this case a preconcentration is necessary before the determination. This is mainly a chemical step, which includes elimination of organic material by ashing, dissolution etc. If the residue from ashing cannot be dissolved easily, a fusion is necessary.

Chemical separation of the element to be determined becomes necessary, when there are interferences from major, minor or trace constituents, including the matrix. Single element separation can be useful, when adequate standards are not available for calibration, which can be done with an aqueous solution of the element concerned.

With increasing sensitivity, selectivity and reliability of the instrumental methods available for the measurement of the analytical signal, the need for chemical steps has diminished. But there is no doubt, that because of the enormous complexity of the analytical problems modern trace element analysis in biological material never can avoid them.

Data processing and evaluation form an integral part of the trace analysis procedure. Modern instrumental

methods, in view of the large volume of information output, require the processing of these data mainly by computerized methods.

Statistical methods are helpful in evaluation of the reproducibility of results and to establish correlations between the data. The more serious problem of systematic errors has to be solved by the use of standard reference materials whose composition matches the sample analyzed, analysis of the same sample by different methods in the same laboratory as well as by intercomparison among different laboratories. The importance of data evaluation in trace element analysis of biological material, which was overlooked to some extent in the past, needs to be emphasised.

Even if we have followed all these steps and rules mentioned above and have done excellent work within each step, it might be possible, that our result still shows errors of more than thousand percent. This might be due to wrong sampling, the analytical sample not being representative of the amount of material to be evaluated. Therefore, more attention has to be paid to sampling and sample preparation.

It is important to formulate at the beginning of any analytical work, a clear and precise question, which is sought to be answered by the final analytical result. However, it may sometimes turn out, that the question cannot be answered by an analytical number at all. The answer to the question will involve statements about the reliability of the analytical data obtained.

1.3 General steps of trace element analysis

Taking into consideration all these aspects, trace element analysis in general, including the more complex cases, involves the following 12 steps (14, 15, 16):

Formulation of the question to be answered

1. Sampling
2. Sample preparation
3. Ashing, fusion
4. Dissolution

5. Preconcentration
6. Separation
7. Preparation of the measuring sample

8. Physical measurement
   integral
   spectral
   calibration

9. Data processing
10. Data evaluation

Answering the question.

In case of activation analysis, nuclear activation is carried out between the steps 2/3 or 6/7/8.

1.4 Presentation of trace analysis data

A most up to date compilation of trace element contents in human tissues is reported in the literature (4).

The presentation of the result of a trace element analysis in terms of contents or concentrations in literature uses quite different expressions. In most cases, contents or concentrations are given as amount of element related to the weight of sample in case of solid samples and related to the volume in case of body fluids.

Therefore, the content of one part per million (ppm) is given as μg/g or mg/kg and one part per billion (10^9) (ppb) as ng/g or μg/kg. Confusion, however, exists about the type of sample weight used. There is a choice between fresh weight, dry weight, ash weight, fat-free dry weight (e.g. muscle), and ash-free dry weight (e.g. bone). However, the first two mentioned are preferred in literature. Major and minor elements are given as percent or equivalents and moles per unit weight. Use of the most simple fresh weight involves errors due to variations of the water content either in the original sample or change during sampling and sample preparation. The dry weight eliminates this difficulty, but sometimes is difficult to determine because of decomposition of the biological material during drying at 105 or 110°C due to hygroscopic properties. Therefore, freeze drying is generally preferred.

In addition, valuable conclusions sometimes can be obtained from the ratio of two or more element contents in the sample, either related to gram, mole or equivalents.

The important aspects of data reduction and data evaluation in trace analysis are only being realized more carefully in recent years. These range from the estimation of parameters such as mean, median, variance etc. and statistical tests to reveal significant differences between different sets of data to more complex aspects of recognising useful patterns in the distribution of trace elements in terms of correlation between elements.

1.5 Trace elements analysed in biomedical samples

According to IYENGAR, KOLLMER, BOWEN (4), about 55 elements have been quite frequently reported in the literature under normal conditions in human tissues. Generally, only 6 to 7 major elements build up the matrix and about 5 others are the main constituents of the body electrolytes. As given in table 1 trace elements can be grouped with some overlapping into essential, non essential, toxic and other elements.

1.6 Classification of biomedical samples according to similar major and minor element composition

The chemical treatment of a biomedical sample during any analytical procedure is determined mainly by its matrix element composition. The matrix of most biomedical tissues is built up by the structural elements C, H, N, O, P, S and in the case of hard tissues also by Ca and to a lesser extent by F. Major elements in the body electrolyte solutions are Ca, Cl, K, Mg and Na. A few matrices can be characterised by higher amounts of the minor elements such as Al, Ba, Cl, Cu, F, Fe, I, K, Mg, Na, Si, Sn, Sr and Zn which can be found at a concentration level of 0.01 % and above. Using the data reported by IYENGAR, KOLLMER, BOWEN (4) body tissues have been classified by IYENGAR and SANSONI in 1976 into groups containing certain elements at a) >1 %, b) 1 to 0.1 % and c) 0.1 to 0.01 % concentration levels. These classifications are given in tables 2 and 3.

Table 1:
Biological classification of the elements found in biomedical material
(V. Iyengar, B. Sansoni, 1976)

Structural elements: C, Ca(*), H, N, O, P, S

Electrolyte elements: Ca, Cl, K, Mg, Na

Trace Elements:

1. **Essential:**

1.1 Biologically important: Co, Cr, Cu, F, Fe, I, Mg, Mn, Mo, Ni,
1.1 Biologically important: Se, Si, Sn, V, Zn
1.2 Clinically significant: (Co), (Cr), Cu, Fe, Mg, (Se), Zn

2. **Suspected to be essential:** As, Ge, Rb

3. **Regularly found in the tissue:** Al, B, Br, Ga, Li, Sc, Sr, Ti

4. **Toxic:**

4.1 Potentially toxic: As, Cd, Hg, Pb, Sb, Se
4.2 Major environmental contaminants: Cd, Hg, Pb
4.3 Industrial hazards: Be, Bi, Cr, Mn, Ni, Sb, Si, Th, Te, U, V, W

5. **Others:** Ag, Au, Ba, Ce, Cs, Nb, Pt, rare earths, Te, W, Zr

6. **Radioactive contaminants(**):** Po, Ra, Rn, Th, U; Am, Cm, Np

*) Bone only;
**) if not listed as elements before.

Table 2:
Analytical Classification of biological materials according to major elemental composition
(V. Iyengar, B. Sansoni, 1976)

Contents: xxx = >.10mg/g dry weight, resp. >.17
Contents: xx = 1–10mg/g dry weight, resp. 0,1–17
Contents: x = 0,1–1mg/g dry weight, resp. 100–1000ppm
without x = <0,1mg/g dry weight, resp. <100ppm

Samples	Ca	Cl	Fe	K	Mg	Na	P	S	Si	Zn	Additional elements
Solids:											
Adrenal	x	xx	x	xx	x	xx	xx	xx	x		
Aorta	xx	xx	x	xx	x	xx	xx	xx	x		
Bone (composite)	xxx	x	x?	xx	xx	xx	xxx	x	–	x	
Brain (composite)	x	xxx?	x	xx	x	xx	xxx?	xx	x		
Breast	x	xx	–	xx		xx	xx	xx			
Esophagus	x	xx	x	xx	x	xx	xx	xx			
Feces (a)	xx	xxx	x	xxx	xx	xx	xxx	xx	x	x	Cu:x; Mn:x; Sn:x; Zr:x; (ICRP)
Gall bladder	x	xx	–	xx	x	xx	xx	xx	–		
Hair	x	xx		x		xx?	x	xxx	x?	x	
Heart	x	xx	x	xxx?	x	xx	xx	xx		x?	
Intestine	x	xx	x	xx	x	xx	xx	xx	x?		
Kidney (composite)	x	xxx?	x	xx	x	xx	xx	xx	x	x	
Larynx	x	xx	x	xx	x	xx	xx	xx	–		
Liver	x	xx	xx	xx	x	xx	xxx?	xx	x?	x	
Lung	x	xx	x	xx	x	xx	xx	xx	x		Al:x
Lymph	x	xx	x	xx	x	xx	xx	xx	xx		Al:x
Muscle (skeletal)	x	xx	x	xxx?	x	xx	xx	xx		x	
Nail	x	xx		xx		xx	x	xx	x	x	Al:x
Ovary	x	xx	x	xx	x	xx	xx	xx			
Pancreas	x	xx	x	xx	x	xx	xx	xx		x	
Placenta	xx	xx	xx	xx	x	xx	xx	xx			
Prostate	x	xx	x	xx	x	xx	xx	xx		x	
Skin (composite)	x	xx		xx	x	xx	xx?	xx	x		
Spleen	x	xx	xx	xx	x	xx	xxx?	xx		x?	
Stomach	x	xx	x	xx	x	xx	xx	xx	x	x	
Testis	x	xx	x	xx	x	xx	xx	xx			
Thymus		x	x	xx	x	xx	xx	xx			
Thyroid	xx?	xx	x	xx		xx	xx	xx		x?	I:xx
Tongue	x	xx	x	xxx	x	xx	xx	xx		x?	
Tooth (composite)	xxx	x		xx	xx	xx	xxx	x	x?	x	Ba:x; F:x; Sr:x?;
Trachea	x	xx	x	xx	x	xx	xx	xx	–		
Urinary bladder	x	xx	x	xx	x	xx	xx	xx			
Uterus	x?	xx	x	xx	x	xx	xx	xx		x?	
Liquids:											
Blood (composite)		xx	x	xx		xx	x	xx			
Erythrocytes		xx	xx	xx		x	x	?			
Serum (human)	x?	xx				xx	x	xx			
Cerebrospinal fluid	xx	xx		x		xx					
Gastric juice			x		xx						
Bile (gallbladder)	xx	xx	x	xxx	xx	xxx	xx	–		x	
Milk (mature) (a)	x	x		x		x	x	x			
Pancreatic juice		xx		x		xx					
Prostatic fluid	xx	xx	–	xx	–	xx	–	–		xx	
Seminal fluid	x	xx	–	xx	x	xx	–	–		x	
Sweat	xx	xx		x	xx	xx	–				
Urine	x?	??	xx	xx	x						

– = contents not available or uncerfain;
? = borderline cases; (all values according to ICRP reference man)

Table 3:
Classification of biomedical samples according to similar major element contents
Data are taken from table 2. Content range 0.1–1 mg/g or ml is listed only for Si and Zn.
(V. Iyengar, B. Sansoni, 1976)

Element content	Ca	Cl	Fe	K	Mg	Na	P	S	Si	Zn
xxx >1 %	bone tooth	brain feces kidney		feces heart muscle tongue bile*		bile*	bone brain feces liver spleen tooth	hair		
xx 0,1–1 %	aorta feces placenta thyroid CFS bile prostatic fluid sweat	all others, excepting bone and milk	liver placenta, spleen, erythro-cytes	all others excepting hair, serum CSF gastric juice milk pancreatic juice	bone feces tooth bile* sweat urine	all others excepting erythro-cytes milk urine	all others excepting hair lung nail; all body fluids (without bile)	all others excepting bone tooth; among fluids only blood and serum	lymph nodes	prostatic fluid
x 100– 1000 ppm	**Remarks:** 1. Feces also: Cu (x); Mn (x); Sn (x); 2. Al (x) in lung, lymph nodes, nail 3. I (x) in thyroid 4. tooth also: Ba (x); F (x); Sr (x?) 5. bone also: F (xx) 6. dry urine also: Cl (xxx); K (xxx); Na (xxx); P (xxx); S (xxx); Ca (xx); 6. dry urine also: Mg (xx); Br (x); Si (x);							(x): adrenal aorta brain feces hair intestine kidney liver lung nail skin stomach tooth	(x): bone feces hair heart kidney liver muscle nail pancreas prostate spleen stomach thyroid tongue tooth uterus	

*Bile taken from gall bladder

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2. SAMPLING OF BIOLOGICAL MATERIAL

2.1 Fundamental aspects

2.1.1 Basic considerations

The totality of a material shall be judged and estimated by the result of a quantitative analysis in terms of concentrations or contents of the components analysed. Only a very small part of this bulk of material can be used for producing the analytical signal, from which contents can be calculated. In general, a large sample (b) is taken from the totality (a) and transported to the laboratory (laboratory sample). Subsequently the sample size has to be reduced to a much smaller analytical sample (c). From this, very often again only a small fraction can be used to produce the analytical signal, the signal sample (d).

Reproducible and reliable analytical results can be expected only, if the smallest signal sample (d) has exactly, or as closely as possible, the same mean composition with respect to the elements to be determined as the totality of material to be estimated. In a first approach, this can be achieved only, if the totality of material (a) is ideally homogenous. By far this is not the case while dealing with most of the solid samples. Therefore it is a good experimental rule, never to expect true homogeneity, until it is established. In a second approach, when the material (a) is heterogenous, sampling statistics leads to several different sampling methods, such as the random, systematic and differentiated sampling. This approach also deals with sampling units, sample size reduction with respect to reduced particle size; optimisation of sample weight, particle size and homogeneity with respect to the precision, manpower and cost of the analytical result.

Because of this situation, as a rule the analyst himself should be involved directly in the sampling procedure. He should take care to ensure representative sampling, elimination of contaminations and losses of constituents to be determined or any changes in the mean composition during sampling, transport and storage. In case of medical restrictions, where the analyst has only limited possibilities to carry out the special sampling procedure, the medical staff should be familar with the requirements of valid sampling.

A reorganisation of the whole analytical approach might be necessary if it is doubtful whether the analytical sample (d) is representative, if the sampling procedure approved by the analyst is not completely followed, or the required sample quantity with desired particle size is not available.

The analyst on his part, should get sufficient background information about the whole analytical problem, the sample history and approximate sample composition before outlining the sampling programme.

Compared with some other fields of material analysis, it appears, that in trace element analysis of biomedical material, not much has been done to systematically apply the sampling statistics.

General Reviews:

Some general information is given for the biological background of trace elements in (15, 34, 69), about trace element analysis in (6, 13, 16, 17, 19, 30, 38, 47, 50, 61, 65, 87, 89, 98, 104, 112, 114, 115, 124, 126) and about sampling in (1, 4, 26, 41, 51, 67, 84, 90, 91, 104, 113).

2.1.2 Fundamental laws

The sample and its subsamples must satisfy several conditions:

The mean composition of a laboratory and analytical sample must in principle be exactly equal to the mean composition of the totality of material to be evaluated *(Representative mean composition).*

The variance within the laboratory and analytical sample should be an unbiased estimate of the variance of the totality of material *(Representative variance).*

The accuracy of all sampling operations together should be at least of the same order of magnitude as the accuracy of the subsequent analytical procedures *(Accuracy).*

It is necessary to compromise between the agreement of the totality and analytical sample with respect to mean composition and variance on one side and a given expenditure of costs and manpower on the other *(Efficiency).*

2.1.3 General sampling methods

Representative samples can be obtained by several methods. In *random sampling,* a number of samples are randomly chosen from the totality of material. This is valid only in case of a truly homogeneous totality of material or in the case of smaller heterogeneous totalities, which can be homogenised before sampling, e.g. liquid samples.

On the other hand, representative samples cannot be obtained by random sampling of inhomogeneous totalities of material. The analytical error in this case largely depends on the degree of inhomogeneity. This method, however, is less problematic for liquids.

In *systematic sampling* a systematic sampling network approach is used. For larger totalities of material which

are impossible or impractical to homogenise in a single batch, this method can be applied, e.g. in population studies for the homogeneous parts of a heterogeneous material.

In *differentiated sampling* of totalities with localized sections, *these nests are analysed.* The composition of the heterogeneous totality is computed, based on the amount of each homogeneous part. Several rules must be obeyed. However, this method is applicable only for larger totalities with visual heterogeneities which are either impossible or impractical to homogenise in a single batch, e.g. a total body analysis.

2.1.4 Sampling units

These are some of the logical sampling elements of a totality and its samples, which have to be considered in the sampling procedure. Two types of materials have to be distinguished. One type of material occurs in discrete portions, which can be specified as sampling units, e.g. beads, corns, pills, stones, teeth, single hair, single man in a whole population. Another type of material contains no unique subdivisions. Most biological materials from one organ and body-fluids e.g. liver, feces, urine, belong to this group.

Only for the first type of material, a routine procedure can be specified, that will ensure a properly unbiased sample.

A more precise result is obtained by mixing groups of units and performing N analyses of the mixed groups than by analyzing the N units separately.

For an analytical method with constant coefficient of variation (standard deviation proportional to the amount of element), the analytical precision is independent of the amount of analytical sample analyzed.

2.1.5 Influence of various parameters on the optimal sample size

The following considerations are valid for solid sampling, especially for subsampling of hard tissues such as bone and tooth.

2.1.5.1 Sample size of particulate matter

In general, the sample weight should increase with the particle size of the material, variation in composition of the sample particles (heterogeneity) and desired accuracy of analysis.

2.1.5.2 Constant number of particles per sample

The totality of material (a) and its subsamples (b) to (d) should have, in principle, the same degree of heterogeneity with respect to their composition of particles at all stages of subdivisions. Therefore, the total number of particles in the sample must be the same in all stages of subdivision. A decrease of sample weight during subsampling, must be accompanied by a corresponding decrease in particle size. The sample weight could be decreased as the cube of the particle diameter. However, at smaller particle sizes, a greater number of particles is required for several reasons. But grinding should not be more than necessary, because of possible changes in composition (see reference (10) in chapter 1). If possible, in trace element analysis grinding should be avoided because of sample contamination.

2.1.5.3 Sample size and number of particles

The number of particles per gram of material increases exponentially with the particle diameter. Because of the strong influence of the increase in particle diameter, it is important to avoid even a few abnormally large particles in a sample. To be on the safe side, the sample size for particulate material should be estimated on the assumption of the maximum particle diameter present. If a constituent to be determined is present only as a small fraction of the totality, for a neglible sampling error, the number of particles (units) required in the sample becomes enormously large (see reference (10) in chapter 1).

2.2 Basic hazards

The basic hazards faced in all the steps of the analytical procedure, are contamination by elements, loss of trace elements and changes in mean composition with respect to elements to be determined.

2.2.1 Contamination by elements

The contamination of the sample by trace elements or even the main constituents during sampling may occur from the environment of the sample, the sampling operation itself and the operating personnel.

In case of biomedical samples, extracellular fluids and other organs and tissues around the sample in the human or animal organism constitute the environment of the sample. Air dust is a common source of contamination.

During the sampling operation, contamination may arise from air dust and volatile contaminants from the air. In addition, the sampling tools may contribute to various trace element contaminations to a marked degree. Numerous possibilities of contamination of the samples by the operating personnel also exist. Exhaled air, spit, phlegm, sweat, cosmetics, tobacco ash, tobacco smoke or even the clothing debris may contribute to contamination. Contamination which may arise from the laboratory atmosphere has been summarized in table 1.

Various metallic implements made of high purity stainless steel and other metals (e.g. titanium) have been useful as controlled contaminants. The main disadvantage of using such implements is that the constituents of these implements cannot be determined in the handled biological material.

The same is true also for implements made from nonmetallic materials. Some examples of very commonly used nonmetallic materials are plastics such as polyethylene, polypropylene, teflon, etc., and inorganic materials such as quartz, agate, corundum, boron nitride which are widely used as materials for constructing mills and grinders. Especially quartz and corundum are available in high purity form which introduces controlled contamination of only a few selected elements such as silicon and aluminium. Trace element impurities found in some commonly used laboratory wares are summarized in table 2.

Yet another form of contamination arises from leaching of the container walls. These include the innumerable varieties of laboratory ware made of glass and plastic. Some examples of leached out impurities from various types of laboratory materials while handling HCl, HNO_3 and HF, are summarized in table 3.

Some chemical reagents are used as stabilizers during sampling operations. Similarly use of preservatives is a major contamination risk, e.g. formalin. All these reagents should be analysed seperately in order to determine the blank value adequately, if use of these reagents is unavoidable. Some of these results are summarized in table 4.

2.2.2 Loss of trace elements

There are several possibilities of loss of trace elements during the sampling procedure.

Volatilisation of some trace elements occur during drying. Even at temperatures as low as 80°C trace elements such as mercury and selenium are lost. At 110°C and above additional losses are reported (see drying under sample preparation).

Adsorption on container walls and tools is another source of error especially for body fluids with very low concentration of trace components. If this adsorption is due to an ion-exchange mechanism, a higher salt concentration or acidity reduces this type of adsorption remarkably. For example in tenth to one normal solutions of salts or strong acids there is only a slight or neglectable adsorption of most trace elements. Several investigators have discussed these problems (3, 7, 8, 9, 10, 11, 25, 35, 36, 37, 39, 54, 55, 82, 100, 102, 103, 108, 111, 119, 120, 121, 122). With occasional exceptions, quartz, teflon and high purity polyethylene containers are most suitable for storing the dilute solutions.

As a general rule, standard and sample solutions should be stored only in a more concentrated form and diluted shortly before use.

Losses of solutions due to sputtering, spraying, agitation or loss of a solid samples e.g. dust or ash during ashing are trivial, but can be observed relatively often. Errors of this kind are however, not reproducible.

In case of sedimentation or precipitation in liquids, trace elements may also be lost by coprecipitation.

Overlooked differences between the chemical state of the trace element present in the sample and the added carrier of the same element might be a serious problem, which may lead to relatively high losses of the trace constituent. The same might be true for differences in the endogenous state present between in vivo and in vitro samples and the inorganic form of a carrier or the added tracer (40, 46, 70, 75).

2.2.3 Change in mean composition

Changes in mean composition of the sample with respect to the trace elements to be determined may interfere severely during sampling and the following analytical operations. Details are discussed under sample preparation.

2.3 Special restrictions

Samples originating from human and animal organisms impose some special restrictions on sampling operation due to various considerations, unlike other biological materials such as plant and food samples. Certain ethical demands make the sampling operations for human samples still more difficult.

2.3.1 Samples of human origin (in vivo samples)

2.3.1.1 Stringent medical preparations

Most organs and tissues from living human organism, especially the solid samples, are to be taken with stringent medical precautions. Sterile conditions are a usual precondition for applying procedures from internal medicine, surgery, gynecology and histology.

Table 1:
Trace element levels and laboratory atmosphere

Element	Non-filtered[1] air	Filtered[1] air	Tobacco[2] smoke	Cosmetics[1]	Sweat[3]	Skin[3]	Hair[3]
	µg/g dust	µg/g dust	µg/g*	µg/g	µg/ml	µg/g wet	µg/g
Al	3000	6				1–2	4–29
As	55	<0.01	2.85			0.06–0.10	0.2–3.7
Br	23	<0.02	71.50	4000	0.2–0.5	4–10	<1–53
Ca	2690	<0.004		$6x10^4$	4–206	250	200–3190
Cd	2.8	0.1				0.032	0.24–2.7
Cl	1.5	<0.005		630	1054–2041	2654	950–4805
Co	9	0.1	0.034	1.12	0.02	0.05	
Cr	39	<0.006	0.35			0.10	0.1–3.6
Cs	1.3	<0.01	0.01			0.03	0.4–1.1
Cu	213	<0.02			0.1–1.5	10–20	11–32
F	1.0	0.10			0.2–2.0		
Fe	3230	<0.006	7.30	1100	0.5–1.5	10	5–68
Hg						0.10	1.3–7.6
I	2.9	<0.01			0.01	0.10	<1–15
K	7920	<0.004		250	176–350	3067	150–663
Mg	2.4	<2390			1–48	58	19-163
Mn	116	<0.006			0.06	0.1	O.3–5.7
Mo						0.05	0.06–0.21
Na	2950	134			1017–3370	2000	18–1720
Ni	70	0.50			0.05–0.2	0.05	0.6–6.5
P	1150	1.50		1400	0.2–1.5	327	83–165
Pb	2150	<0.04			0.05–2.7	0.10	3–70
Rb	23	<0.01				2	0.2–0.50
S	20000	<0.003		400		1500	47700
Sb	14.8	<0.03	0.15			0.04	0.1–3
Se	0.6	<0.02	0.22			0.25	0.6–2.53
Si			$9x10^4$			100–200	20–1950
Sn	9.6	<0.05					?
Sr	13.5	<0.01				0.10	0.05–0.92
Th							?
Ti	258	3		6300		0.50	0.05–14
Tl						0.03	0.012
U	0.1	<0.01					0.00013
V	259	0.005				0.15	0.005–0.53
Zn	1640	<0.02	10	$3.5x10^4$	1	6–20	99–450

* condensate [1),2),3)] ref. 47, 93a, 69, respectively

Table 2:
Trace element impurities in laboratory ware materials

Element	Glass	Polyethylene (high pressure)	Process unknown	Tygon	Plexi glass	Synthetic quartz	Teflon
	µg/g	ng/g	ng/g	µg/g	ng/g	ng/g	ng/g
Al	100000[1]	80–3100	230–3000	55			
As						0.17	
Br			186			70	
Ca	1000[1]	200–20000	200	5			
Cd						0.38	
Cl		1600	800–3000			3700	
Co	0.082	5	0.07–0.31		0.05	0.33	0.33–1.70
Cr		15–300	19–76	6	10	1.60	ND[3]–30
Cs	0.12		0.O5		0.06	0.12	0.05
Cu		4	6.60–17	10	9.50	2.00	22
Fe	3000[1],280[2]	600–2100	10500	50	140	160	ND[3]–35
Hg						0.03	
K	3000[1]			ND[3]		500	ND[3]
Mg	600[1]	80–1500		120			
Mn	1000[1]	10	510	2	32		
Mo						0.40	
Na	300000[1]	170–10000	44–25000	ND[3]			2500–5000
Ni				200		50	
Pb		200	200	200			
Rb						2.43	ND[3]
Sb	2.92	5	0.18		0.01	0.40–3.80	ND[3]
Se			43		0.002	0.70	ND[3]
Si	400000[1]	2000	2000	100			
Sr		800					
Th			3			0.46	
U			840				
Zn	0.73	90	28	5	10	34	8

[1] Pyrex [2] Borosilicate [3] ND = Not detected

Data pooled from 26, 52, 68, 72, 86, 99, 100, 110, 112 and 112a

Table 3:
*Contamination of some mineral acids by leaching of container walls during evaporation concentration in ng/ml and *) in ng/g*

Element Leached	HCl					HNO_3			HF	
	Glass	Poly-ethylene	Poly-propylene	Teflon	Quartz	Poly-ethylene *)	Teflon	Quartz	Poly-propylene	Teflon[1] *)
Al	10	3	0.1	0.3	10		2	20	0.54	3
Br						38				
Ca				5	60		4	60		1
Cd			0.03						0.0007	
Co				0.02		2.6	0.2			
Cr		0.02	0.03	0.9	0.6		7			0.4
Cu	3	4	0.01		1	160	0.01		0.2	0.4
Fe		0.7	0.6	4	10		14	20	0.3	3
Mg	7		0.02	3	10		7	20	0.12	3
Mn		0.005	0.001	0.5	0.4	50	0.2	0.6		0.1
Na	90	2			30	7.5				
Ni		0.03	0.01	0.3			1.0	ND[1]	0.02	0.4
Pb	2.30		0.06	440	0.5		ND[1]	1	0.03	0.1
Si	30	0.8	0.4	1			8			
Sn					0.4					ND[1]
Ti		0.2	0.07	0.04	2					2
V							0.76			
Zn		7	0.02	0.04			0.04		0.1	

[1] ND = not detected

Data pooled from 23, 72, 87, 114a, 124

Table 4:
Impurities in preservatives and intravenous solutions

Element	Heparin[1]	Formalin[2]	Sterile H_2O[2]	0.45 % NaCl[2]	5 % Dextrose[2]
Ba	2.5–12				
Br		0.07			
Ca	300–2900		0.006	ND[3]	ND[3]
Cl		13			
Cr		0.21	(Cu)	0.65	0.36
Fe		3.50			
Hg		0.01			
I		0.07			
K		60			
Mg		1.60	ND[3]	0.0022	0.0014
Mn	3.60	0.09		0.0004	0.008
Na		18			
P		2.40			
Rb		0.04			
Sb		0.14			
Se		0.03			
Sr	5–92				
Zn	28	6.20	0.11	0.11	0.0014

[1] µg/g [2] µg/ml [3] ND = not detected

Data pooled from 15, 71 and 105

Internal medicine procedures have to be used for sampling of most body fluids as cerebrospinal, interestinal, pancreatic, prostatic and synovial fluids, bile and gastric juice.

Surgical procedures are necessary for excision of tumor samples, biopsy of bone, liver, lung, muscle, prostate, skin and for excision of teeth.

Gynecological procedures are necessary for sampling e.g. amniotic fluid, placenta and fetal samples.

2.3.1.2 Legal regulations for autopsy (in vitro samples)

For sampling of material from the dead organism, instead of medical precautions, the usual legal regulations for autopsy have to be observed.

2.3.1.3 Without stringent medical precautions

Several samples can be taken from the living human organism without any medical precautions. Besides hair and nail, the body fluids milk, phlegm, saliva, semen, sputum, sweat, tears as also feces belong to this group.

2.3.1.4 Physiological and pathological influences

Due to the physiological or pathological influences in living human and animal organism, special conditions are necessary for sampling.

Time dependence. Generally, for long time sampling from the living organism, the periodic cycles of sampling may or may not be identical with periodic cycles of the living organism. The conditions to be selected depend on the medical problem. In order to take care of the physiological and pathological influences, sometimes it is necessary, to choose smaller sampling periods of time than the cycles of the organism under investigation or to take samples continuously over a much larger period.

Examples of time dependence of sampling are the diurnal and circadion variations as for example 24-hour collections of urine, blood or feces.

Pre- and post natal state. Examples are the colostrum, mature, transitional state of milk.

Age dependency. Several element concentrations in the body change significantly with age. For comparison of populations, therefore, one has to select the subjects from same age groups. Examples are the embryonic, new born, pediatric, adult and aged state of an organism.

Similar conditions may arise under dietary conditions (e.g. vegetarian, non vegetarian, special diets), stress and related conditions, the position of the sampled subjects (lying, sitting, standing), habits and related aspects (smoking, alcoholism, medication, drug addiction, doping), climatic conditions (tropic, subtropic, temperate, arctic). Random conditions, however should be avoided.

2.3.2 Samples of animal origin

As in human organism, sampling of parts of the living animal body is also governed by certain restrictions. Because of somewhat less ethical and legal considerations, however, the restrictions for autopsy are not valid. Most of the physiological and pathological influences are valid also for the animal body.

2.3.3 Other biological samples

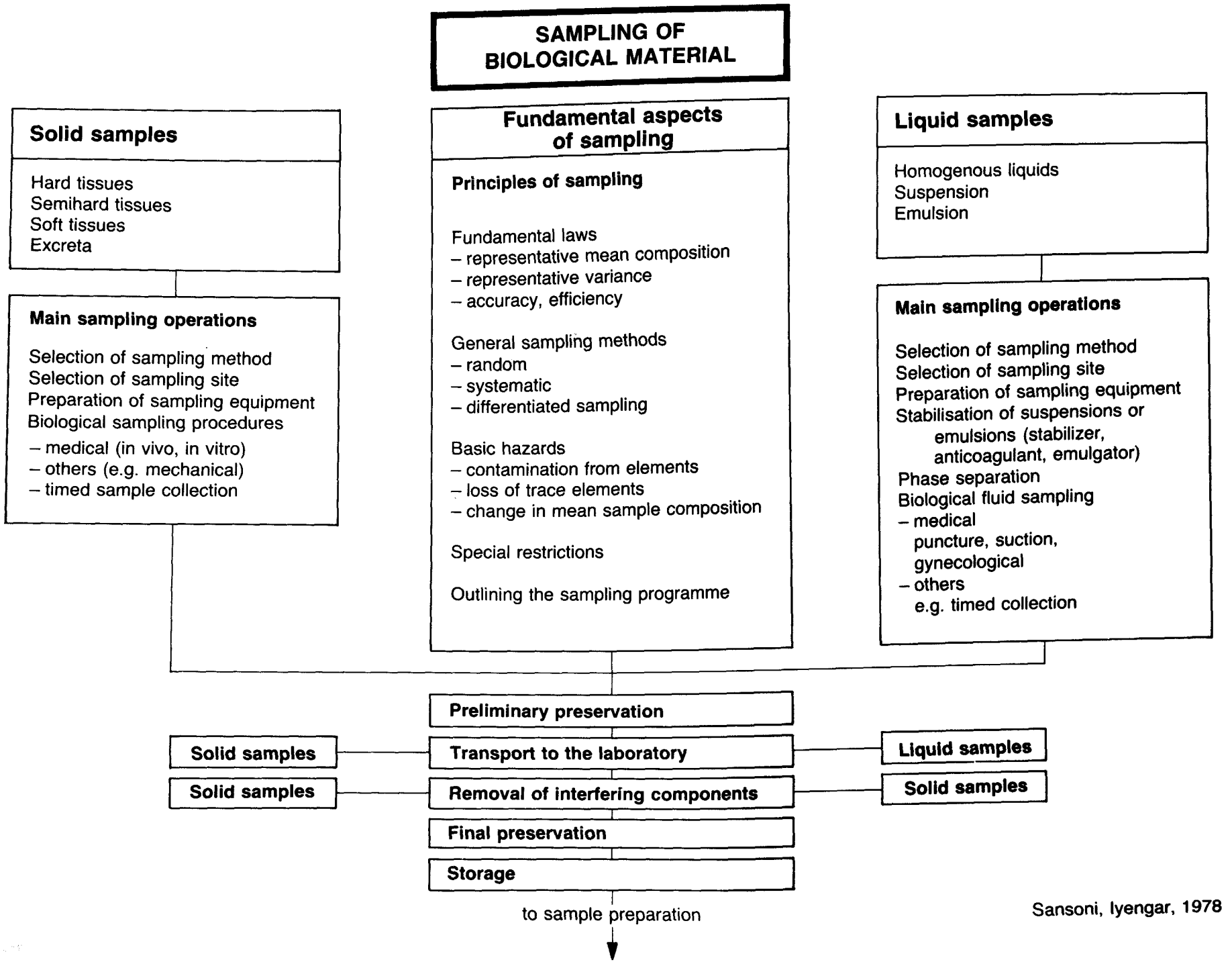
Other biological samples such as most plant and food materials are not governed by medical restrictions. Living plants, however, may also show some physiological and pathological influences on the trace element content. In addition, soil and climatic conditions are of importance in this case. Plant sampling, therefore, should be accompanied in several cases by soil sampling in the same area. Food materials for human use have to conform to severe legal regulations.

2.4 Outlining the sampling program

The initial step of sampling is to establish the sampling plan. The following points have to be considered: – Information about the analytical problem, – Definition of the totality of material to be evaluated, – Special areas of the material under consideration, e.g. bulk/ surface composition, local/topographic distribution, composition of the total material, – Background information about the probable chemical composition of matrix and trace element composition, – Degree of homogeneity to be expected, – Type of homogeneity tests, if necessary, – Sample size in relation to particle size, number of samples necessary in order to obtain a defined accuracy of the analytical result; even using sampling statistics, this can be done often only semiquantitatively or qualitatively, – Consideration of the sample types, – Consideration of special restrictions, – Evaluating the general sampling method to be used, – Additional special considerations, e.g. maximum time the sample can be stored without a change in mean composition etc., – Outlining of the special sampling procedure, considering a compromise between optimal conditions and smallest efforts or costs. The general sampling scheme is given on the next page.

2.5 Solid biomedical samples

Solid biomedical samples are generally heterogeneous. They can be classified with respect to sampling as: Hard



Sansoni, Iyengar, 1978

tissues, e.g. bone, tooth (figure 1), semihard tissues, e.g. nail, hair (figure 2), soft tissues, e.g. most of the body organs and tissues (figure 3) and excreta, e.g. feces

Compared with body fluids, solids have the advantage of low mobility and chemical reactivity. Therefore the danger of contamination from container walls or sample environment and of changes in the mean composition are lower than for the fluids. A general disadvantage is the problem of inhomogeneity and the difficulty in getting truly representative samples.

2.6 Sampling procedures for solids

Sampling of solids is often more complex than sampling of pure liquids. Homogeneity of pure liquids and to a great extent of emulsions and suspensions after stirring and agitation is nearly ideal. With solids, as a general rule, homogeneity of the totality and subsamples should never be expected, until it is proved. Because of the lower reactivity of solids the dangers of contamination e.g. from container walls, losses of trace elements e.g. due to adsorption on container walls and changes in mean composition are generally smaller, as compared with liquids. Sample size reduction is easier for liquids and can be done merely by aliquoting with simple pipettes, burettes or dispensers.

The most adequate general sampling method can be evaluated according to 1.3 depending upon the nature of the sample, limitations by equipment and manpower.

The site or topological situation for sampling and subsampling has to be selected carefully. Examples are different regions of kidney (cortex, medula), muscle (smooth, skeletal, striated), brain, intestine, bone (marrow) hardparts, skin (dermis/epidermis), tooth (enamel/dentin), blood vessels (intima/media/adventitia), hair (distance from scalp), placenta (term of pregnancy), fat (visible, endogenous). Some of these problems are discussed in the following reports (19, 33, 41, 53, 57, 58, 60, 63, 67, 77).

Internal medicine procedures have to be carried out by trained medical staff who are aware of trace element problems.

Cleaning of the surface of samples and subsamples is accomplished by washing, trimming or freeze drying and scraping. Only the non- representative parts of the sample shall be removed considering the basic hazards, special restrictions and special procedures.

Considering the special restrictions, e.g. timed collection of urine, feces, blood etc. may be necessary.

Special and well established procedures, generally in use, are discussed in chapter 3 on sample preparation.

2.7 Liquid biomedical samples

Body fluids are generally heterogeneous, though at first sight, they seem to be homogeneous as for example urine, tears, sweat etc. But they contain in most cases more or less solid cell fragments or crystalline particles.

These heterogeneous fluids are either emulsions or, in most cases, suspensions.

Examples of emulsions are milk, serum (with respect to emulsified fat) and seminal plasma. Mostly all others are suspensions.

An almost comprehensive list of body fluids is given in figure 4.

As pointed out earlier, compared with solids, liquid biomedical samples have the advantage of by far better homogeneity, which facilitates representative sampling by simple methods such as aliquoting stirred solutions. A general disadvantage, however, is the higher mobility and chemical reactivity of the liquid.

2.8 Sampling procedures for liquids

In evaluating the optimal sampling method, it has to be decided, whether a suspension or emulsion, is to be sampled as a whole or the phases have to be sampled separately. Emulsifier in case of milk, stabilizers or anticoagulants (heparin or citrate) in case of blood have sometimes to be added before sampling the whole emulsion or suspension.

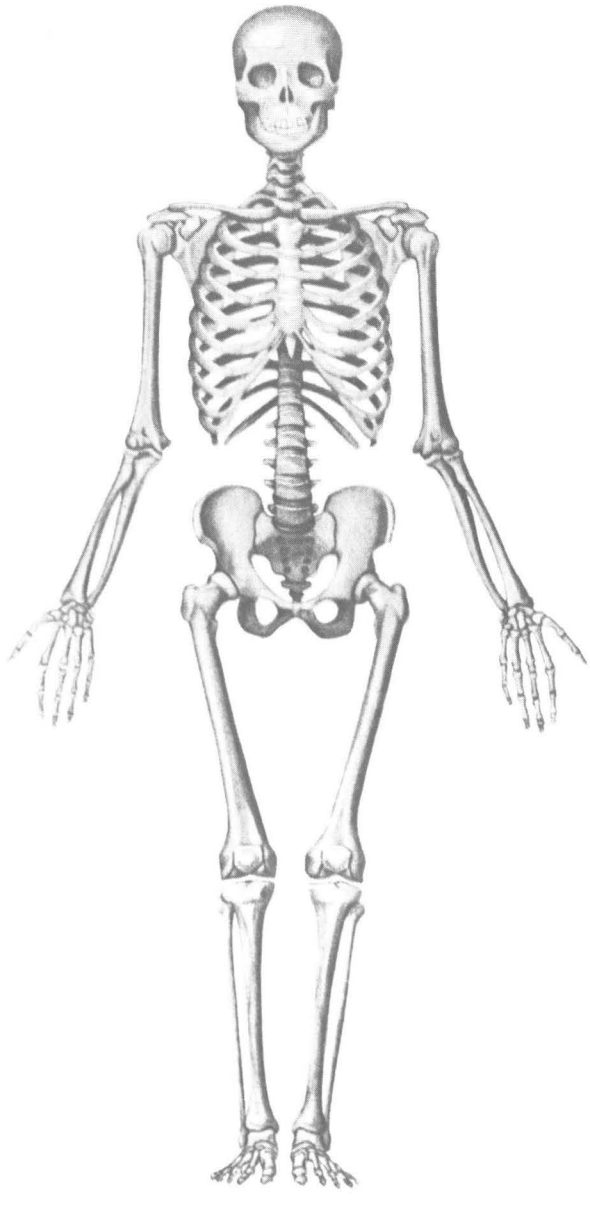
The sampling procedure outlined should take care of the special restrictions if any and the basic hazards in addition to the general aspects. For liquids the problems of heterogeneity and sampling statistics (1.2) are generally less difficult than for solids because of the relatively high degree of homogeneity of pure liquids.

Among medical procedures, internal medicine procedures are used for sampling of e.g. cerebrospinal, intestinal, pancreatic, synovial fluids, taken by syringe and needle; bile and gastric juice by tube (2, 74, 81). Gynecological procedures are employed for e.g. amniotic or fetal fluids, taken also by syringe and needle (special puncture techniques).

If necessary, the two phases of suspensions or emulsions should be separated and weighed before analysis, in order to determine their element content separately and arrive at the values for the whole sample.

Timed collection is necessary for several body fluids, for example a 24 h cycle be followed during urine sampling (27).

Physiological and pathological factors also have to be considered.



HARD TISSUES

Tooth:

Enamel
Dentin

Stones:

Gall–
Bladder
Kidney

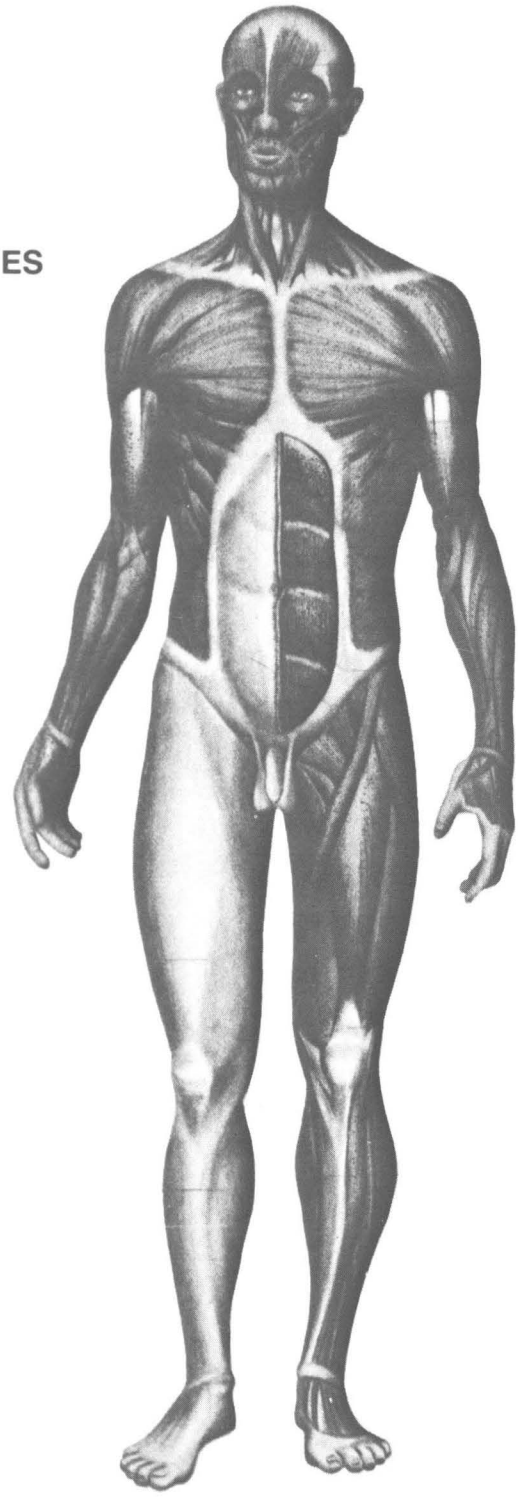
Bone:

Hard part
(Marrow part)
(Cartilage)
(Tendon)
(Connective tissue)

KFA ZCH	**HUMAN SAMPLES** Hard tissues	ly, Sa 1978 Figure 1

SEMIHARD TISSUES

Hair
Nail
Cartilage
Tendon



SOFT TISSUES

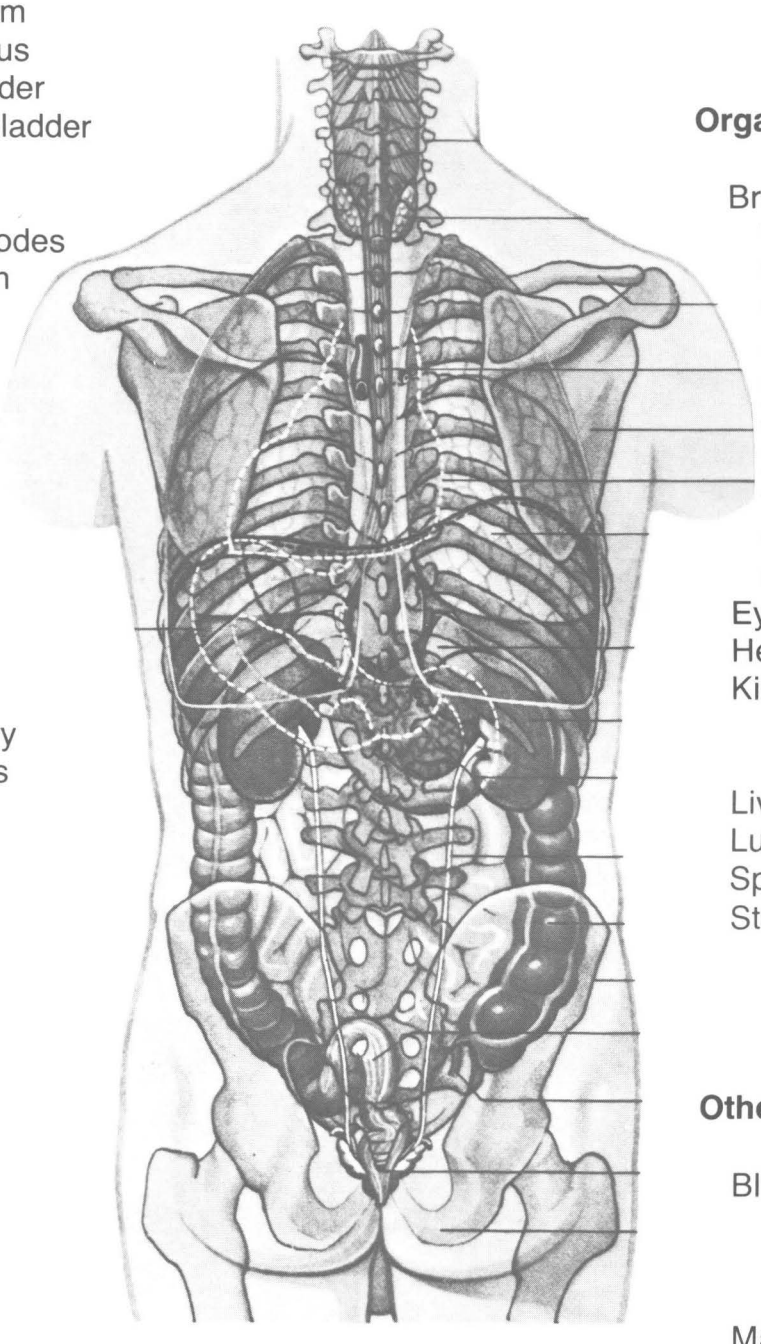
Major tissues:

Muscle
Smooth
Skeletal
Striated
Skin
Dermis
Epidermis

KFA ZCH	**HUMAN SAMPLES** Semihard and major soft tissues	ly, Sa 1978 Figure 2

Minor tissues:

Diaphragm
Esophagus
Gall bladder
Urinary bladder
Intestine
Larynx
Lymph nodes
Omentum
Thymus
Tongue
Trachea
Urethra



Organs:

Brain
- White matter
- Grey matter
- Prosencephalon with telencephalon and diencephalon;
- Rhombencephalon with mes-, metmylencephalon; spinal cord

Eyes
Heart
Kidney
- Cortex
- Medula

Liver
Lung
Spleen
Stomach

Glands:

Adrenal
Mammary
Pancreas
Pituitary
Prostate
Salivary
Thyroid

Other soft tissues:

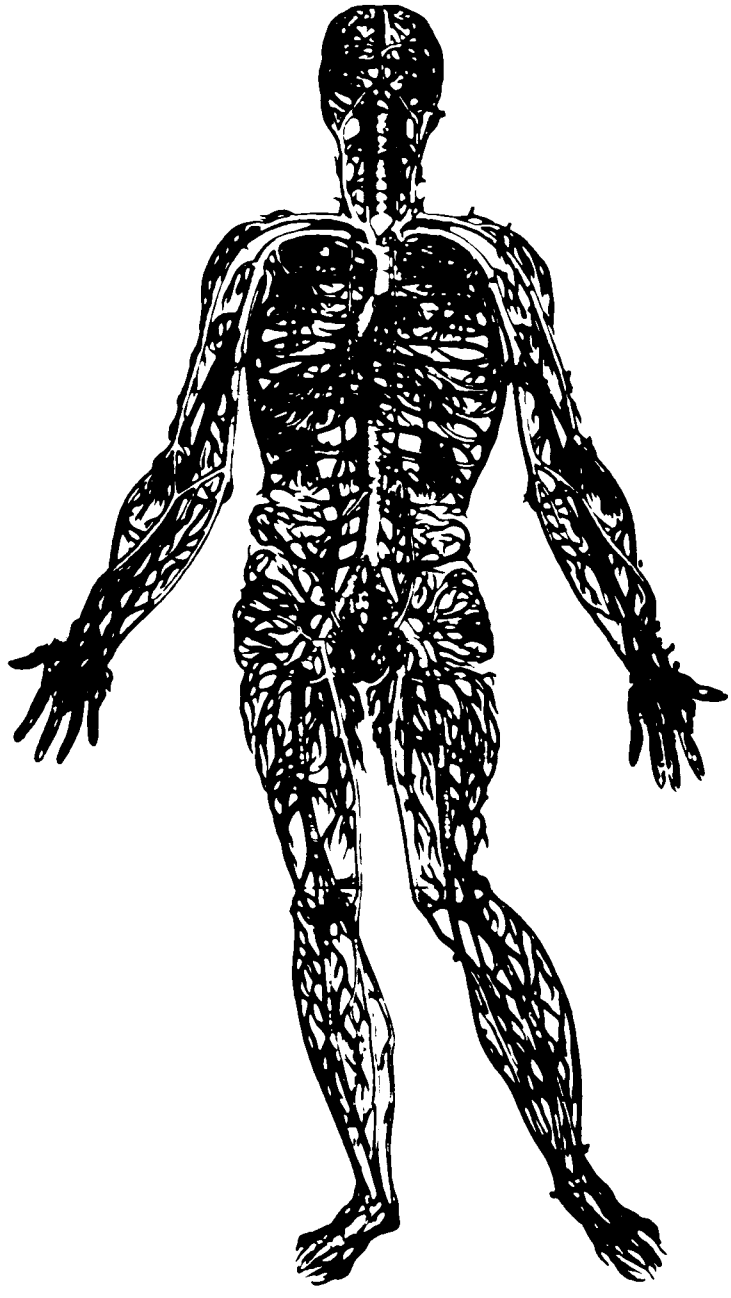
Blood vessels
- Aorta, Intima, Media, Adventitia

Marrow part and
Connective tissue from bone
Fat
Nerves
Placenta

Reproductive organs:

Ovary
Ovum
Testis
Uterus

KFA ZCH	**HUMAN SAMPLES** Soft tissues	ly, Sa 1978 Figure 3



Body fluids
and related
components

Aqueous humor
Blood (whole)
- Erythrocytes
- Leucocytes
- Plasma
- Serum

Cerebrospinal fluid
Edema
Fetal fluids
- Allantoic fluid
- Amniotic fluid

Intestinal fluids
- Cecal fluid
- Duodenal secretion
- Ileal serection
- Jejunal secrection

Gastric juice
Bile
- Gallbladder
- Hepatic

Milk
- Mature
- Colostrum
- Transitional

Pancreatic juice
Pericordial fluid
Peritoneal fluid
Pleural fluid
Phlegm
Prostatic fluid
Saliva
Seminal fluid
- Seminal plasma
- Sperm

Sputum
Sweat
Synovial fluid
Tears
Transudates
Urine

KFA ZCH	**HUMAN SAMPLES** Body fluids and related components	ly, Sa 1978 Figure 4

2.9 Preliminary preservation

For most biomedical samples, which need to be stored for several days, because of microbial attack and chemical decay at least some preliminary preservation before transport to the laboratory and sample preparation is necessary or at least to be recommended (20, 29, 31, 43, 76, 78). The procedures are very similar for solids and liquids. Heterogeneous liquids may need the addition of emulsifiers, stabilizers or anticoagulants.

General preservation such as freezing or cooling to 2-4°C is advisable. Thawing after deep freezing, however may give rise to concentration gradients.

The nature of additives and the amount added, should be such, that practically no element contamination and no change in the mean composition of the sample occurs.

Minimisation of humidity and air dust in the environment is to be recommended.

2.10 Transport to the laboratory

Most of the factors are the same for transport of both solids and liquids. As a general rule, as with all analytical steps, there shall be no contamination or loss of constituents and no change in mean composition of the sample.

Transport, therefore, should be as quick as possible, at lower temperatures (4°C) or in a deep frozen state in special containers (for transport up to 4 days) if necessary, under exclusion of air dust, humidity and sometimes also air; without contamination from the container material, losses of trace elements by evaporation or adsorption on the container walls. Therefore, proper packing is very important.

Especially for solids, segregation should be avoided or if this is not possible, after transport repeated homogenisation is necessary.

Whenever possible, whole organs should be transported with their own capsules.

Sensitive liquids have to be transported with a minimum of turbulence. Use of containers with non-wettable surface as e.g. teflon, reduces the dangers connected with liquid-container wall interactions. Blood samples for serum separation should be transported within a few hours. Generally, it is a rule of thumb to centrifuge them within one hour.

2.11 Removal of interfering components

Interfering components of the sample are such parts, which do not belong to the totality of material (body organ or fluid etc.) to be evaluated. They may contribute to a wrong mean composition of the sample and must therefore be removed before analysis. The operations are different for solids and liquids and some tissues are so intricately mixed with their sample environment, that complete elimination of the interferences without affecting the integrity of the tissue itself is not possible. A good example is that of residual blood in blood rich organs, e.g. liver and placenta. Some of these problems are discussed in the following reports (29, 47, 64, 65, 68, 71a, 80, 81, 84).

2.11.1 Solids

There are several interfering components within the sample, which have to be removed. They shall only be listed here: Connective tissues, capsules, skin (separation of dermis from epidermis), visible fat, blood vessels, nerves, hair (for skin sampling), marrow from bone, tooth (enamel from dentin), blood vessels (separation of intima, media, adventitia, sclerotic plaques), glandular parts, intestinal content from the gastro-intestinal tract (e.g. feces, remnants of food, soil, residual blood), extracellular fluids; excess of blood, fluid and other tissues from placenta; avoiding contamination of feces samples with urine or blood from menstruation or internal bleeding.

Some of these procedures can be carried out by the analyst, the main part, however, only by the medical staff.

2.11.2 Liquid samples

Since all body liquids are aqueous and miscible, only removal of solid particles has to be considered here.

Removal of cells and other particles in e.g. urine, sweat, bile can be achieved by centrifugation, sedimentation, to some extent filtration and by cell electrophoresis; separation of blood cellular components from each others, e.g. erythrocytes, leucocytes and thrombocytes (platelets) by differential centrifugation; removal of residual erythrocytes from serum by repeated centrifugation.

The interference from fat in whole blood, serum and plasma can sometimes be reduced by sampling under dietary control and adequate timing.

2.12 Final preservation

The final preservation for storage of the sample has to be done in the same way as the preliminary preservation according to 2.2. The only additional possibility for solids is sterilisation by gamma ray irradiation.

2.13 Storage of samples

2.13.1 Purpose

Storage of the sample obtained after the sampling procedure may have several purposes. The sample has to be preserved for sample preparation and the following analytical steps, if these cannot be made immediately after sampling. In long term investigations, the samples can be collected and analyzed afterwards in one series. It may be necessary to prepare and store counter samples, which are identically prepared aliquots, one of which remains with the user of the analytical data for independent cross checking if necessary, e.g. food samples. In cases of reconfirmation or doubtful results, the analysis of a counter sample stored in the laboratory has to be repeated after a period of time. Storage of counter samples for a definite time interval is necessary sometimes in forensic and clinical analysis for legal reasons. Standard and standard reference materials are produced often in very large quantities which have to be stored for years. A last argument for long time storage is the preservation of characteristic ecological, environmental or biological samples for future generations for reference monitoring.

2.13.2 General considerations

During storage, contamination of the sample from container walls and the whole sample environment, trace element losses and changes in mean composition have to be prevented (2, 3, 17, 27, 39, 44, 51, 65, 83, 86, 100).

Containers with non-wettable walls (teflon, polyethylene etc.) are useful. Surface preconditioning especially of quartz and glass containers can be done by mineral acids such as HNO_3, HCl and diluted HF, by chelating reagents (EDTA) or oxidants (H_2O_2), followed by careful rinsing with thrice or five-times distilled water from quartz stills and then steaming (39, 54, 59, 101).

The container material and cleaning procedure should be specified and documented. Because of the basic hazards, generally the storage time should be kept as short as possible. If necessary, samples should be prepared again. In general, storage time is a function of the organ or tissue in question, the speed of tissue autolysis and sometimes intestinal flora attack of the organism. The storing temperature should be as low as possible. The frozen state for fresh organs or wet samples is generally preferable. The total surface area of the container and the free space in it should be minimized. Containers with non-porous, smoth and non-wettable surfaces are often preferable. As far as possible, whole organs or tissues should be stored without dividing them into smaller parts.

The larger the amount of sample stored in a bottle, the less significant will be the influence of container surface.

2.13.3 Packing

Proper packing helps to protect the sample against the basic hazards. A wide variety of containers (bottles, boxes, cans, etc.) with different container materials (plastics, mineral materials, metals) can be used. Protection from water vapour and air is important. Diffusion through the container walls especially in case of plastic walls has to be considered in some cases. Packing in two or even three containers and wrapping with plastic foils can be helpful.

2.13.4 Storage in preservative solutions

Preservation of whole organs and tissues can be accomplished by storing within preservative solutions. Commonly used are formalin and alcohol. This type of storage however, has severe disadvantages for trace element analysis. Trace elements can be eluted into the solution and lost. Trace elements from the solution can be taken up by the sample; blanks of the solution, therefore, have to be analysed additionally. Changes in mean composition, leaching of elements from the container walls, osmotic translocation and cell breaking can very easily occur in the liquid system. Usually a mixture of 70 % ethanol and 15 % aqueous formalin (v/v) is used.

2.13.5 Container material

The container materials are listed below in decreasing order of importance in each group.

Polymers: Polyfluorohydrocarbons (teflon, kel-F, tetzel, halar etc.), polyethylene (high pressure PE generally prefered over low pressure PE), polypropylene (hostalen, hostaflon etc.), silicone rubber (is one of the purest rubbers, but contamination risk by Zn is reported), polymethyl methacrylate (plexiglas, perspex; relatively low in trace element impurities).

Ultrasilica (synthetic quartz), carbonglass; borosilicate glass. Metals: High purity aluminium foil or container, platinum, high purity titanium etc.

2.13.6 Freezing

Under ideal conditions, freezing is the best long term preservation method. For short term storage often temperatures of about 2 to 4 °C under normal refrigeration are enough. Long term storage should be done generally by deep freezing at -20 °C. However, during freezing, because of denaturation of proteins and redistribution of elements due to rupture of cell walls by ice crystals, some irreversible processes may take place.

2.13.7 Drying

Freeze drying provides a good mode of preservation since the elimination of water and denaturation of proteins destroy enzymes and bacteria. The volume reduction results in preconcentration.

Disadvantages of oven drying are the loss of a few volatile trace elements due to matrix decomposition, e.g. Hg and Se (40, 46, 70, 75). Dried tissues cannot be reconstituted, unlike frozen tissue. Drying at lower temperatures requires longer exposure of the sample to the drying environment.

2.13.8 Storage of solids

Storage of solids generally is less dangerous than storage of liquids because of their lower chemical reactivity.

2.14 Literature

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3. SAMPLE PREPARATION OF BIOLOGICAL MATERIAL FOR TRACE ELEMENT ANALYSIS

3.1. General considerations

3.1.1 Principle

The trace element content of a given element in a biological material is calculated from the intensity of a suitable analytical signal originating from that element. Aquisition of a satisfactory analytical signal involves as preceding steps, ashing for the elimination of the interfering organic matrix, preconcentration in order to improve sensitivity, separation to eliminate interferences from other elements within the sample and preparation of the subsample used for the analytical signal. It should therefore be kept in mind that sample preparation discussed here is only a precursor to the other operations. Nevertheless, practice has shown that in most cases of trace analysis both sampling and sample preparation are extremely important steps. Any inadequacy at this stage results in basic errors which may not be compensated by the following steps and amount to more than one order of magnitude in terms of total analytical error.

The sample obtained by a sampling operation has to be reduced in its amount during subsampling in the laboratory into much smaller analytical samples for measuring the analytical signal. In the case of solid samples, particle size reduction, homogenisation and homogeneity tests are necessary before subsampling. For subsequent pure instrumental measurement some sort of a preconcentration is at times useful.

Body fluids are generally heterogeneous suspensions or emulsions. In this case either the phase separation into pure liquid and solid phase or stabilization and homogenisation of the suspension or the emulsion, followed by homogeneity test have to be considered. If any preconcentration step is used, rehomogenisation and homogeneity test should be done. Both solid and liquid samples have to be preserved in order to avoid changes in their composition with respect to the elements to be determined, if not analysed immediately. Ashing and dissolution are regarded as different steps after the sample preparation and are discussed in section 4.

Based on the above arguments, the generalised sample preparation scheme presented in figure 1 has been developed. Because of the complexity of materials and situations a perfect general scheme valid for all the cases is not possible. Changes may be necessary in several cases. Therefore, subsampling has been discussed here as well as under sampling. The sequence of operations consisting of particle size reduction, homogenisation, homogeneity test and subsampling may have to be changed or repeated. It has been attempted to make this review as general as possible. Therefore, it may be used as a "check list" for the trace element analysis of biological material.

3.1.2 Requirements

A well defined analytical programme, prior knowledge of the approximate elemental composition of the sample matrix and a reasonably clean working bench justifying the concentration levels sought in the sample, are some of the basic requirements. For example, the elements Cu, Fe and Zn are present in ppm levels in most of the biological samples and require only a reasonably clean laboratory to prepare the samples. At the other extreme is the preparation of blood serum samples for Mn analysis. The concentration of Mn in blood serum is 0.6 ng/ml and unusual precautions are necessary to avoid external contamination of the sample (122), since Mn is generally present in the environment e.g. in air dust.

Unlike the basic sampling operation which may at times preclude the direct participation of the analyst, e.g. autopsy regulations, medical restrictions and lack of sufficient biological background, the responsibility of preparing the samples for the analysis belongs entirely to the analyst. Hence it should be possible in a reasonably well organized laboratory to assess the basic hazards such as contamination, loss and changes in the mean composition at every stage of the sample manipulation. However, any scope for an elaborate sample preparation by particle size reduction, preconcentration, homogenisation and subsampling comes into picture only when there is enough sample material and the analyst is not confronted with small quantities such as those obtained by needle biopsies.

For *liquids* as a general rule, the total number of liquid transfers should be kept to a minimum and only accessorries made of non-wettable and low impurity construction materials should be used.

Handling of *solid* samples demands more stringent conditions. Problems of contamination and loss of the elements are imminent at every stage of the analysis. They demand a minimum of sample manipulation and therefore require adequate and suitable equipments. Since these samples are exposed to ambient atmosphere for different periods of time, dust free containment is a basic necessity at all stages of the preparation. The magnitude of the induced contamination and its prevention is dependent upon the element involved. For example Mn, Si, Pb, Zn and Cu create more problems than Pu, U, Th, Bi, Tl and Te. Air borne contamination is especially dangerous for Pb analysis at low concentrations. Another potential source of danger during the sample preparation is the cross contamination from the interfering components, e.g. contamination of serum Fe by hemolysis, contamination of muscle Pb from skin which contains higher Pb (72).

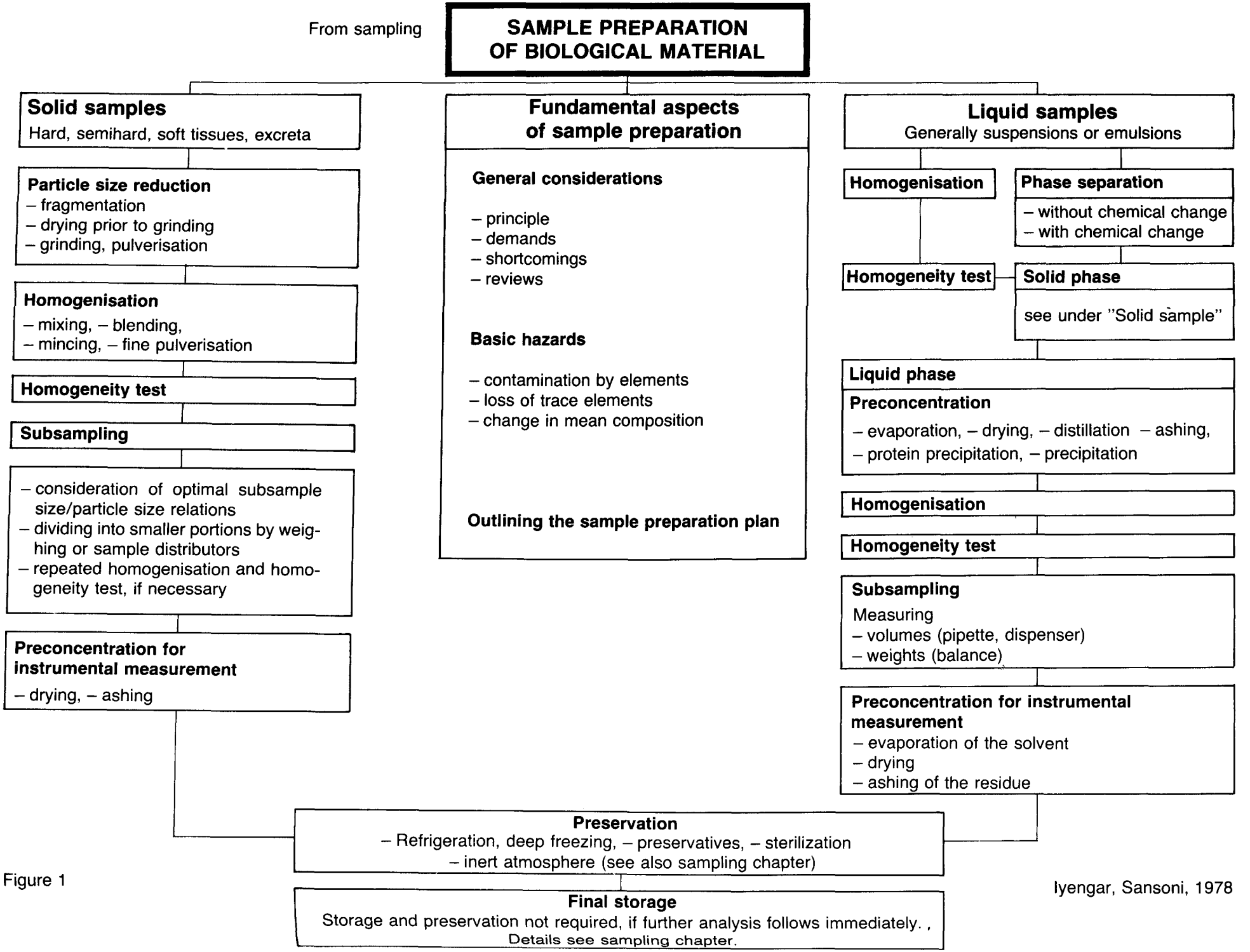


Figure 1

Iyengar, Sansoni, 1978

3.1.3 Shortcomings

There are many predictable and unpredictable sources of error during the preparation of biological samples for trace element analysis. These are chiefly lack of recommended standard procedures for tissue handling, the working environment, practical limitations during sampling and subsequent sample handling, the chemicals, the apparatus, and finally the analyst himself.

Among these are a few factors over which the analyst has little or no control, e.g. medical restrictions, inability to eliminate residual blood and extracellular fluids from complex organs without affecting the authenticity of the sample and lack of standardized equipments for tissue handling. The magnitude of such errors is dependent upon the element of interest, the sample size and the method chosen for the analysis, e.g. contamination from reagents and analytical blanks especially at very low concentration levels. Hence sampling with the subsequent preparation stages is often very difficult to achieve with accuracy and precision, and continues to pose a difficult challenge to the analyst attempting to prepare representative samples or the sub-samples.

Thus personal involvement of the analyst in identifying and assessing some of these limitations is an essential part of the basic requirements.

3.1.4 Reviews and selected references

A few comprehensive reports discuss the numerous problems in preparing biological samples for trace element analysis. They include general discussions on contamination (7, 8, 98, 99, 111, 125, 129, 137), sample handling and treatment (1, 3, 54, 55, 91, 94, 124, 129), handling of liquid samples (5), destruction of organic matter (4, 18, 49, 50, 81, 106), specimen collection and preservation (2, 113), homogeneity (19, 20, 68, 69, 120), special problems concerning selected samples such as urine (32) blood serum (22), liver (69), kidney (89), thyroid (83), hair (9, 121), biopsy of muscle (16) cerebrospinal fluid (79), saliva (119) and sweat (126).

3.2 Basic Hazards

Contamination by elements from outside, loss of trace elements from the sample and changes in the mean composition of the sample with respect to the elements to be analysed, plague the trace element analysis at all stages starting from sampling to measurement. But sampling and sample preparation are especially sensitive and susceptible to these basic hazards.

3.2.1 Contamination by elements

The *working atmosphere* in the laboratory during sample preparation contributes to the contamination.

Reagents are a major source of contamination depending upon their purity, the concentration level of the element analyzed and the method of analysis to be used for a sample. Examples of trace element impurities, in different types of water samples and reagents analysed by various investigators are presented in table 1.

Among *sample handling equipments*, of fundamental importance are clean benches and glove boxes, which are unavoidable at least for working in the ppb range. Unfortunately, not all the laboratories presently reporting data at this concentration range are suited for ppb level competence (see sampling chart in sampling chapter).

In-vitro or autopsy materials provide better opportunities to prepare samples with controlled contamination using tools made of quartz, plastic or boron nitride. With the possible exception of elements such as Mn, Cr and Ni in certain samples, high purity steel knives not freshly sharpened have been found to be satisfactory for sample preparation (117). High purity titanium has been used by some investigators and seems to be a promising solution. Disposable plastic hand gloves, teflon tweezers, polyethylene, teflon foils and wax paper (para film) are the other handy aids. Since any sample handling equipment is a potential source of some contamination, it depends on the purpose of the analysis, if a compromising tool can be found. This difficulty is especially seen in biopsy samples. If optimal materials for sample preparation tools are not available, it is necessary to check the possible contaminations under realistic conditions.

In general, for *liquids*, because of higher mobility, reactivity and surface wetting, the contamination problems are more serious than for solids. The various construction materials used for manufacturing syringes, sample collecting tubes, needles, stoppers (especially rubber), pipettes, dispensers, diluters, preconcentration tools and storage containers are all sources of possible contamination. Since needles made of rare alloys such as Pt-Ir and Pt-Rh are not economically feasible for routine use, high purity stainless steel needle preferably with a plastic catheter appears to be the practical alternative if its limitation with respect to Mn and Cr is kept in mind (122). Use of disposable plastic tools have been finding increased application in this connection.

For *solids*, sources of contamination by elements may arise from dust and volatile contaminants in the laboratory air; sweat, cosmetics, tobacco ash, exhaled air, phlegm from operating personnel, laboratory ware, sample preparation tools and chalk dust, debris from clothes and wiping paper, preservatives such as for-

Table 1:
Trace element impurities in some reagents used for sample preparation.
Concentrations in µg/l

Element	H_2O			HCl		HF		HNO_3		H_2SO_4		$HClO_4$	
	Tap[a]	Demine-ralised	Single[b] distil-lation	p.a.	ultra pure	p.a.	ultra pure	p.a.	ultra pure	p.a.	ultra pure	p.a.	ultra pure
Al	57	0.10	< 0.002	8	0.80	4	0.5	7	1	8	–	–	–
As	–	–	–	–	–	–	–	–	0.005	–	–	–	–
Br	95	0.10	–	–	2.60	–	–	–	7	–	–	–	–
Ca	55000	1	< 0.0003	72	0.30	0.4	52	0.2	0.4	10	2	760	0.2
Cd	0.70	< 0.10	< 0.007	0.03	0.003	8	0.005	0.1	0.03	< 1	< 1	0.1	0.05
Cl	14100	1	< 0.0004	–	–	–	–	–	–	–	–	–	–
Co	–	< 0.10	0.02	0.09	0.001	< 1	1	0.018	0.01	< 1	< 1	–	–
Cr	–	< 0.10	0.0002	1.10	0.008	5	0.6	72	0.10	25	2	10	9
Cs	0.02	–	<0.00001	0.002	< 0.002	–	–	< 0.01	< 0.1	–	–	–	–
Cu	–	0.20	< 0.002	0.20	0.03	0.50	0.30	1.30	0.2	3	3	11	0.10
F	1.40	–	< 0.0002	–	–	–	–	–	–	–	–	–	–
Fe	–	0.20	< 0.0005	1	–	60	0.60	1300	0.80	8	–	330	2
Hg	–	< 1	–	–	–	< 10	< 10	–	–	< 10	–	–	–
I	9.40	–	< 0.001	–	–	–	–	–	–	–	–	–	–
K	28000	0.04	< 0.0001	200	0.10	0.40	1	< 10	9	< 10	4	200	0.6
Mg	10400	0.30	< 0.0002	7	0.30	2	0.1	3	0.40	3.30	2	500	0.2
Mn	2.20	0.05	< 0.0005	< 2	0.001	0.60	0.03	9	2	8	0.8	–	–
Mo	–	0.02	–	0.02	–	–	–	–	–	–	–	–	–
Na	8100	0.03	< 0.0002	500	0.20	100	0.60	80	0.01	20	9	600	2
Ni	30	< 0.1	< 0.0002	0.20	0.005	0.50	0.05	0.70	0.03	< 1	0.20	8	0.50
P	43	0.004	< 0.0003	–	0.20	–	7	0.80	0.50	–	–	–	–
Pb	8.50	0.10	< 0.003	0.20	0.0015	2.20	0.002	0.20	0.01	1.2	1	2	0.20
Rb	10	–	< 0.001	–	–	–	–	–	–	–	–	–	–
S	14100	4	< 0.0003	–	3	–	–	0.60	15	–	–	–	–
Sb	0.60	< 0.50	< 0.002	0.20	0.38	–	3.0	0.03	0.04	–	–	–	–
Se	3.30	–	–	–	–	–	–	0.20	0.09	–	200	–	–
Si	4900	0.50	–	20	1	–	4	30	8	18	–	–	–
Sn	0.60	0.10	< 0.004	0.07	0.002	11	0.05	0.10	0.002	0.60	0.20	0.30	0.30
Sr	11000	0.06	< 0.007	2	0.06	0.50	0.10	0.20	0.01	0.40	0.30	14	0.02
Th	–	–	< 0.0002	–	–	–	–	–	–	–	–	–	–
Ti	–	< 0.1	–	–	0.006	–	0.50	0.50	0.80	–	–	–	–
Tl	–	–	< 0.0001	0.10	0.10	0.20	0.10	0.20	–	0.10	0.10	0.10	0.10
U	–	–	–	–	–	–	–	–	0.003	–	–	–	–
V	18.50	< 0.1	0.40	–	0.08	–	–	0.05	–	< 2.40	–	–	–
Zn	5.60	< 0.1	< 0.002	1	0.03	6	0.10	4	0.08	< 1	< 1	7	0.10

a) Tap water from UK, Belmont area, Survey
b) Thermal destillation in quartz and then passed through a double stage mixed bed ion exchange followed by filtration through a complex teflon filter.

NH_3/H_2O (p.a.) = < 0.0003 Sc; < 0.04 Cr; < 0.1 Fe; 0.009 Co; 6,0 Cu; 2,3 Zn; < 0.1 Ag; < 0.006 Sb; < 0.002 Cs (ppb) (112)

30 % H_2O_2 = p.a. = 0.003 % residue; 0.003 % H_2SO_4; 0.8 ppm Cl; < 2 ppm SO_4; < 3 ppm PO_4; < 2 ppm N; < 0.02 ppm Pb; < 0.02 ppm Cu; < 0.02 ppm Ni; < 0.1 ppm Fe (114)
DAB VI = 900 ppb sum of heavy elements including 200 ppb Fe

Data pooled from (17, 37, 54, 60, 67, 78, 84, 95, 100, 101, 102, 105, 108, 112, 124, 139)

malin and alcohol, stabilizers e.g. for preserving the viability of platelets. Particle size reduction, preconcentration and homogenisation are more dangerous steps than preservation and final storage. This is especially significant for hard tissues such as bone and tooth.

More details about contamination from various sources are given in chapter 2 on sampling.

In the case of neutron activation analysis, virtual contamination can arise from the interfering nuclear reactions, which either increase or decrease the number and therefore the concentration of the radioisotope used for element determination. Because of interfering (n,α) and (n,p) reactions by fast neutrons, too high values for the elements P, Si and Cr are obtained due to the interfering reactions $^{35}Cl(n,\alpha)^{32}P$, $^{32}S(n,p)^{32}P$, $^{34}S(n,\alpha)^{31}Si$, $^{31}P(n,p)^{31}Si$ and $^{54}Fe(n,\alpha)^{51}Cr$. For equal thermal neutron and fission neutron flux, the interference of Fe in the determination of Cr in serum and whole blood was shown to introduce an analytical error of 3 and 650 % (!) respectively (103). Another typical example is that of the determination of P in blood serum.

An additional positive error may result by the overlapping of analytical signals from two or more elements at the same time. Examples are some peaks of gamma ray spectrum in activation analysis, e.g. (a) ^{76}As (559 keV), ^{82}Br (554 keV), ^{124}Sb (564 keV), (b) ^{75}Se (280 keV), ^{203}Hg (279 keV), (c) ^{46}Sc (1120 keV), ^{65}Zn (1115 keV) and (d) ^{64}Cu (511 keV) interfered by other positron emitters and high energy gamma rays, e.g. ^{24}Na.

3.2.2 *Loss of trace elements*

The most harzadous steps for the loss of trace elements are ashing, drying, evaporation to dryness and in case of liquids surface adsorption on container walls, tools and suspended solid particles.

The volatility of an element depends to a large extent on its chemical form in the sample. For example, molecular ($Hg(CH_3)_2$) or metallic (Hg) mercury is much more volatile than ionic (Hg^{2+}, $HgCl^{2-}$) mercury.

In general, with respect to volatilisation losses, no or little difficulties are caused by Ac, B, Ba, Ca, Mg, Mn, Mo, Nb, Pa, rare earths, Sc, Cr, Ta, Th, U, V, W, Y; problems arise only during dry ashing for Ag, Be, Cd, Co, Cs, Cu, Fe, I, Ir, K, Li, Na, Ni, Pd, Pt, Rb, Rh, Sb, Sn, Tl, Zn. Whereas I, Po, Se, Te, Tc, Re, Ru, Os are volatile under oxidizing conditions in aqueous solutions. Volatile even in boiling hydrochloric or hydrobromic acid are As, Ge, Hg, Sb, Sn (50).

During oven drying it has been shown, that metabolized elements for example Hg from liver or Sb from whole blood are volatile to some extent even below 105°C (79). More details about volatility of trace elements can be found under ashing and drying.

Losses of elements due to adsorption on container walls can be serious especially for liquids. They depend mainly on the chemical form and concentration of the trace element, pH and salt concentration of the solution and the type of the container material. These losses decrease generally with increasing element concentration, decreasing pH and increasing salt concentration. The best suited container materials are teflon, quartz and polyethylene. More details can be found under sampling.

In addition, there are several other uncontrolled losses by e.g. sputtering, spraying and mechanical loss of ash during or after dry ashing.

Overlooked differences in the chemical form between the trace element under inverstigation and an added carrier may also result in losses, e.g. As(III/V), Fe(II/III) etc.

3.2.3 *Change in mean composition*

The changes in the mean trace element composition of the sample may result from physical or chemical changes and by redistribution of elements.

The change in sample weight by loss of water is an important source of error, if no special precautions are taken. This is a difficulty often experienced with biopsy samples of soft tissues. The necessary precautions are storage in closed systems, freezing at the sampling site, repeated weighing of the sample as a function of time and extrapolating the weight to the sampling time. On the other hand, residue from ashing and evaporation may absorb water from the surroundings, e.g. lyophylised urine.

Segregation of a heterogeneous mixture of solid particles of different size, shape and density, can contribute considerably; the same is true with changes in particle size.

During reconstitution of frozen fluids by thawing, the protein part tends to form small lumps resulting in concentration gradients if not mixed thoroughly. It has been shown, that alkaline phosphatase content changes in thawed serum samples (22, 25, 30). Since many metals are present as proteins and components of specific enzymes, inhomogeneity in the fluid will affect the accuracy of the determination.

Chemical changes affecting the mean composition may be due to changes in the chemical form of an element during ashing and dissolution, where all the organic forms of the desired elements are converted to inorganic forms (nitrates, carbonates, oxides, etc.). It is conceivable, that during cutting, crushing and grinding due to relatively high mechanical and thermal energies imparted to the sample material, changes may be introduced due to dehydration, oxidation and changes in the

crystal lattice of hard tissues, e.g. charring of bone samples while cutting with laser beam. It is advisable to do these operations under cooling and in a closed system.

Other forms of chemical changes are hydrolysis, oxidation (e.g. decomposition of meat resulting in a colour change from red to grey), hemolysis of whole blood, denaturation of proteins by excess heating or chemical reagents, fermentation, photochemical reactions and microbial attack, e.g. fungus growth.

Possible redistribution of the elements to be determined within different parts of the sample may occur in autopsy samples due to the stoppage of the metabolic functions, which may introduce differences between in vivo and in vitro samples. In case of suspensions, adsorption of trace elements on solid phases may also change the mean composition of the liquid and solid phase to be analyzed.

Low temperature, exclusion of air and light as well as working under sterile conditions or addition of preservatives may eliminate or at least reduce some of these errors.

3.3 Outlining the sample preparation plan

Of fundamental importance is the choice of appropriate sample preparation steps. The number of operations should be minimized and each operation should be simplified to the extent possible. It depends on sampling statistics (sample amount versus particle size), if particle size reduction, homogenisation and reduction in the amount of sample material (subsampling) has to be repeated. The effectiveness of homogenisation should be checked by a homogeneity test where necessary and feasible. When obtaining the subsample for the analytical signal measurement, sampling statistics as discussed under the sampling chapter has to be considered. In several pure instrumental methods for trace element analysis, simple drying is sufficient for preconcentration without any other chemical operation.

When preparing liquid samples, it has to be decided, whether the analysis should be carried out for the heterogeneous mixture or the separated solid and liquid phases independently.

A primary requirement for establishing the sample preparation plan is also to minimize the basic hazards as discussed earlier.

3.4 Sample preparation of solid samples

The sample preparation of solid biological material involves particle size reduction, homogenisation and homogeneity test, subsampling and subsequent preservation and final storage. However, in practice, it is not always possible to strictly isolate one step from the other.

3.4.1 Particle size reduction

Particle size reduction is part of sample preparation as well as of sampling. In order to represent the true mean composition and variance of the totality of the material to be estimated, relatively large samples should be taken in the field or hospital. However the analytical sample, which is used for producing the analytical signal, has to be much smaller. Therefore dividing or splitting of the original sample during subsampling into several smaller samples is unavoidable.

Both original and analytical samples have to be representative with respect to the mean composition of trace elements to be determined as well as to its variance. According to sampling statistics (see sampling chapter), the smallest amount possible for the analytical sample depends on the maximum particle size of the laboratory sample. The smaller the analytical sample taken, correspondingly smaller should be the particle size.

For this reason, in most cases particle size reduction is an unavoidable step before subsampling. An additional purpose of particle size reduction is, to promote subsequent dissolution of solid analytical samples by enlarging their surface. Furthermore, several instrumental methods such as X-ray fluorescence analysis or alpha spectrometry prefer small particle size because of the smaller self absorption within single grains.

Particle size reduction is an important step before subsampling not only for hard, but also for soft tissue.

3.4.1.1 Methods for hard and semi hard tissues

Operations for particle size reduction of hard and semi hard tissues such as bone, tooth, nail, hair etc. are fracturing, crushing, grinding and fine pulverisation. In addition, soft tissues can be converted into hard and brittle solids by deep freezing at temperatures around that of liquid nitrogen. Best known examples are the fine pulverisation of hair and nail in a vibration mill (69).

Equipment for fracturing, grinding and pulverisation. Fracturing of hard tissues such as bone or tooth can be achieved by simple sawing with a stainless steel or diamond saw. A primitive, but effective way is to wrap it up with polyethylene foil and break with the help of a bench vice.

Coarse crushing into particles with diameters down to 1 or 2 mm can be done in a jaw crusher or an old fashioned steel mortar. Chips can be achieved with a turning lathe. However, all these operations are prone to contaminate the sample by the equipment material.

Concerning grinding and pulverisation, choice of the type of grinder or mill depends mainly upon the brittleness, hardness, cleavage, compressive strength of the

sample material, the maximum particle size of the sample before and after grinding, the amount of material, the time available, the tolerable temperature and type of atmosphere to be used during grinding and last but not least upon the abraded grinder material getting into the sample.

Sample materials with a hardness (Mohs) from 8.5 to 4.5 or even 2.5, such as bone, tooth and residues from dry ashing can be ground or pulverised more easily than semi hard (hair, nail) or fibrous (plants) and elastic (connective tissue) samples. The latter group needs either special types of grinders and mills or preferably grinding at temperatures of liquid nitrogen as mentioned before.

Suitable for grinding hard to semi hard materials are for example the jaw crusher, mortar grinder, planetary speed mill, centrifugal ball mill, vibration ball mill. Semihard to soft fibrous and elastic materials, including dried meat, fish or plants may be ground better in an ultra centrifugal mill, cross beater mill or a vibration mill. Suitable for grinding at low temperatures after cooling in liquid nitrogen is especially the vibration ball mill (68).

Construction material of the grinder. One has to accept, that during any particle size reduction, especially during fracturing, crushing, grinding and pulverisation, the sample becomes more or less contaminated by the grinding material of the apparatus. To overcome these difficulties, there are two different ways:

a) quantitative determination of the amount of abrasion by analysis of a main element of the grinder material in the sample, if it is absent in the sample material before grinding;
b) use of high purity grinding material without other major, minor and even trace elements; the element itself shall not be analysed in the treated sample.

Most promising for the future might be the second way (b). Until now, however, only a few metals, e.g. such as high purity titanium and nickel have been used. Among the non-metallic grinder materials, alumina and agate seem to have the smallest element contaminations. Quartz is not hard enough for many purposes and also very brittle.

In any case, the complete chemical composition of major, minor and even trace elements in the construction materials of crusher, grinder and mills, which come into contact with the sample, should be known. Some manufacturers have provided this information (140) as mentioned below.

Agate (from Brazil or Uruguay): 99.91 % SiO_2; 0.02 % Al_2O_3; 0.02 % Na_2O; 0.01 % Fe_2O_3; 0.01 % K_2O; 0.01 % MnO; 0.01 % CaO; 0.01 % MgO; hardness (Mohs) 6.5 to 7.
Sintered alumina: 99.7 % Al_2O_3; hardness 9.
Chrome steel (Nr. 1.2080): 85.3 % Fe; 12.0 % Cr; 2.1 % C; 0.3 % Mn; 0.3 % Si.
Chrome nickel steel (Nr. 1.4112): 77.85 % Fe; 18.0 % Cr; 1.15 % Mo; 0.85 % C; 1.0 % Mn; 1.0 % Si.
Hard metal (tungsten carbide, WC+Co): 87 % W; 5.7–5.8 % C; 5.6–6.0 % Co; 0.7–1.0 % (Ti+Ta); 0.1 % Fe; hardness 8.5.

Abrasion of grinder material. The abrasion of grinder material in contact with the sample increases in the following order: hard metal, agate, sinter alumina, chrome and chrome nickel steel, hard porcelain. The abrasion of agate is about 250 times smaller than that of hard porcelain (140).

The following list summarises the elements for which contamination from structural materials of a grinder can result. Extensive contamination has been observed with Co, Cr, Fe, Mn, Mo, Nb, Ni, Ta, V, W from stainless steel; moderate contamination by Al, Cu, Mg, Mn, Na and Sc from aluminium; Al, B, K, Na, Si from pyrex glass; Mg, S, Sb, Zn from rubber; low contamination by Al, Cl, Ti, Zn from polyethylene; very low contamination by F from polytetrafluoroethylene (teflon) (21a).

During grinding of SiO_2 and $CaCO_3$ (spectral pure) with several mills and mortars under defined conditions, the following levels of contamination have been introduced from the construction material of the mills and mortars into the sample (0.5 g ground from 100 to < 200 mesh, for (a) to (e) below (81) (124a).

a) From agate and poly metharcrylate (PMA): almost no contamination except 2 ppm B and 1 ppm Cu from agate and 4 ppm Mn and 2 ppm Y from PMA into silica (124a) (81)
b) From boron carbide: B 23, Cu 3, Zn 2, Zr 15 ppm into silica and B 13, Y 3 ppm into calcium carbonate (124a) (81).
c) From tungsten carbide: Co 32, Cu 2, Ti 124 and Zn 2 ppm into silica; Co 61, Cu 9, Fe 5, Ni 3, Ti 96 and Zn 6 ppm into calcium carbonate (124a) (81).
d) From synthetic alumina (corundum) Al > 2050, Cr 225, Fe 9, Ga 3 and Mn 1 ppm into silica; Al 452, Cr 5, Mn 3, Y 2 and Zr 15 ppm into calcium carbonate (124a) (81).
e) From alumina ceramics: Al > 2000, Cu 3, Fe 34, Ga 21, Ti 11, Zn 3 ppm into silica; Al > 2050, B 9, Ba 9, Co 65, Cu 7, Fe 130, Ga 31, Li 4, Mn 19 and Zr 14 ppm into calcium carbonate (124a) (81).
f) From silicon: Al 1, Ca 0,71, Cu 0,063, Fe 0,63, Mg 0,95, Ni 0,11 ppm into silica (138a).
g) From agate: Al 2,2, Ca 1,6, Cu 0,131, Fe 0,14, Mg 1,6 and Ni 0,14 ppm into silica (138a).
h) From quartz: Al 4,4, Ca 3,1, Cu 0,074, Fe nil, Mg 0,01 and Ni 0,83 ppm into silica (138a).
i) From plattner mortar: Cr 0,4, Cu 0,35, Se 28, Mn 1,8 and Ni 0,25 ppm into quartz (123a).
j) From molybdenum: Mo 1 % into silica (98a).

Cleaning of the mill. Contamination of the sample may arise by rests of ground material remaining in the mill from preceding grindings. Washing of the grinder by

rinsing with demineralized water is not always possible. In this case some mechanical washing by grinding a portion of the same material or even pure quartz sand before grinding the sample can be useful.

Increasing difficulties in cleaning of the mill are experienced in the order: mortar grinder, vibration ball mill, vibration mill, centrifugal ball mill, planetary speed mill, ultra centrifugal mill, rotor beater mill, jaw crusher, cross beater mill.

Special demands. Loss of sample material within the mill during grinding can be minimized by the use of a centrifugal ball mill, planetary speed mill, vibration mill, mortar grinder and ultra centrifugal mill.

Homogenisation during grinding and pulverisation, especially after addition of small grinding balls with low density, is optimal in the vibration mill, centrifugal ball mill, planetary speed mill and mortar grinder.

Heating during grinding and pulverisation can be reduced by adding an inert organic solvent into the capsule of the vibrator mill. Contamination by trace elements from the solvent however has to be considered.

Water uptake in case of hygroscopic materials can be avoided by exclusion of air in the air-tight grinding insel of a centrifugal ball mill, vibration mill and planetary speed mill.

Cooling during grinding can be done using a coolant in the centrifugal ball mill; an air stream in the cross beater mill and rotor beater mill; solid carbon dioxide in the rotor beater mill and ultra centrifugal mill; or by precooling the sample within the teflon capsule of a vibration mill in liquid nitrogen.

Grinding of elastic plant material can be done with the aid of quartz sand in a mortar mill.

Conclusion. In general, one should avoid grinding of hard tissue as far as possible, since there is the risk of contamination from grinder material. Large samples without particle size reduction are preferred for dissolution. If grinding is unavoidable, then careful hand grinding using an agate mortar sometimes is recommended (90).

3.4.1.2 Soft tissues

Particle size reduction of soft or semi soft tissues is more simple than crushing or grinding hard materials. Main operations are cutting, mincing, blending and ultrasonic homogenisation. Main construction materials are ultrapure quartz (ultra sil), plastics including teflon, polyethylene, high purity titanium and for limited applications stainless steel.

Cutting can be done by a knife, scalpel or a scissors. The usual stainless steel blades, however, may contaminate the sample by mechanical abrasion or even dissolution of traces of metallic elements. These instruments should be made from quartz or plastic in case of knife and scalpel, scissors from plastic or high purity titanium. As yet, these are not commercially available.

For mincing and blending, a variety of different types of apparatus are available for household use, but not much for trace element analysis. Plastic beakers are preferable to glass ones. A serious problem of blenders is the use of stainless steel to manufacture the rotating blades and the shaft.

Ultrasonic homogenisers usually have a lid made of titatnium steel. During ultrasonic treatment, metallic particles from this lid may be sputtered into the sample. These homogenisers are useful for the disintegration of cells in the biological tissues.

3.4.1.3 Drying before particle size reduction

Drying is often necessary before particle size reductions such as grinding or pulverisation. Details are given in the section on drying.

3.4.2 Homogenisation

Homogenisation facilitates subsampling with uniform distribution of the trace elements to be analyzed. It is also necessary for (a) preparing biological standard reference materials, (b) subdividing samples for comparing different analytical methods, (c) preventing the segregation of coarse particles such as dry blood, (d) preparing sample pellets for emission spectrography and spark source mass spectrometry, e.g. mixing with graphite powder. It is recommended further to overcome (e) the natural biological variation of the trace element distribution in the samples and (f) the practical limitations in handling complex samples such as placenta.

Aliquots of varying amounts at a given particle size range should be analysed in order to determine the minimum size of the sample at a known concentration range for a particular element. For example, 0.5 g samples of leaf powders with particle diameters below 220 µm were found to be homogeneous for Ti at 7.3 ppm and As at As 0.87 ppm levels (120).

In an experiment designed to observe the natural distribution profile of certain selected bulk and trace elements no heterogeneity was found for the bulk elements Cl, K, Na and P in bovine liver. However, the trace elements Ag, Co, Fe, Mn, Rb, Sb and Zn showed variations up to ±26 per cent from 6 determinations for sample weights of 47–60 mg (dry) for the bulk elements and 160–260 mg for the trace elements. In order to minimize sample manipulation, instrumental neutron activation

analysis was used. Following homogenisation to a particle size ranging from 40 to 200 μm for the same sample size the maximum variation observed was less than ± 6 per cent (71). These observations are shown in Fig. 2.

The basic requirements for the *choice of the homogenizer*, namely, the use of non-metallic construction material and the need for minimum handling of the sample impose several restrictions on the selection of the sampling and preparation equipments. Since some type of contamination from the homogeniser accessories and grinding has to be anticipated, the grinder material should be carefully chosen in such a way, that it does not contain elements, which have to be determined in the sample. High purity titanium offers a promising application in addition to ultrapure quartz (38), teflon (68), polymethyl methacrylate (21) and high pressure polyethylene.

Liquids do not pose much of heterogeneity problem if sampled by vigorously shaking for a few minutes just before withdrawing the aliquots. However, emulsified liquids such as milk present some problems by forming phases on standing for a long time or due to temperature differences.

For *solid* samples, the problem of homogenisation reaches different dimensions, depending upon whether it is a hard, semihard or soft tissue. Soft tissues can be homogenised in a number of ways without great difficulties especially when they are freeze-dried. However, for tissues such as bone, tooth, hair and nail, use of non-metallic component homogenizers has been rather limited. A recently developed method using brittle fracture technique has been used to pulverize practically all types of biological samples (68, 69). In this method, the sample material is enclosed in a teflon vessel along with a teflon or quartz ball. The vessel is cooled under liquid nitrogen for a few minutes and then fixed between the groves of a Mikro-Dismembrator (Braun Melsungen, Germany) and vibrated for a minute to yield fine powder. Using this method hair has also been homogenised, which is difficult to pulverize by other suitable operations.

3.4.3 *Homogeneity tests*

In order to confirm that the sample has been effectively homogenized, homogeneity tests are necessary. They have to be carried out for a sufficient number of locations of the sample. These tests include determination of certain selected elements at different concentration levels, e.g. K, Zn, Se, Ag which represent major, minor, trace and ultra trace levels. For examining different locations on the surface chosen randomly, electron and ion microprobes can be used. It is also possible to measure the bulk density at different locations of the sample by examining the degree and rate of weight loss during drying, determining the constancy of the ash and potassium content at different locations (20).

For powders, however, uniformity of the particle size can be conveniently checked by sieving and observing the different sieved fractions. Microphotography of powders may also be used to reveal the coarse particles, if any.

The results from homogeneity tests can be statistically evaluated by t-, F-tests and variance analysis.

3.4.4 *Subsampling*

Subsampling means dividing a large sample into smaller portions. It has to be repeated if necessary, until the sample size necessary for the analytical measurement is reached. According to sampling statistics, an optimal ratio of subsample and particle size has to be considered. Partitioning into the required sample amounts can be done either by weighing or using sample distributors. If necessary, pulverisation, homogenisation of the subsamples and homogeneity tests have to be done.

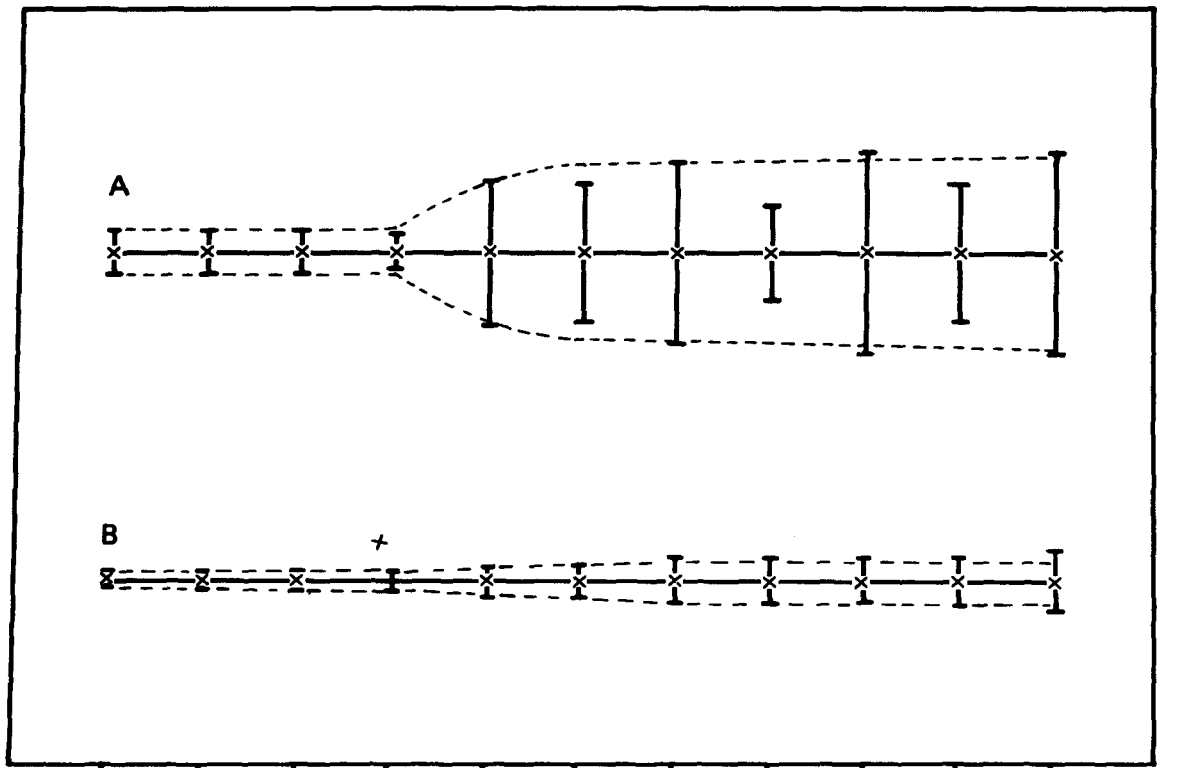
3.4.5 *Preparation of solid samples for instrumental measurement*

For instrumental measurement of solids not requiring dissolution, preparation can be done by simple drying in air, oven or under vacuum or by freeze drying and ashing. These operations also preconcentrate to some extent. Air drying is not common for biological samples. Among the other methods of drying, both oven and freeze drying will be discussed in detail in this report.

During *oven drying* it is important to control the temperature for biological samples. At temperatures around 100°C and above the biological matrix may decompose depending upon the nature of the sample (20). This results in the loss of residual dry matter and intrinsically volatile elements such as Hg may be lost. In an oven drying experiment measuring the dehydration rates in urine, whole blood, serum, erythrocytes, muscle and liver the loss of dry residue observed was most pronounced for urine, which was found to be 31 % (70). The losses from blood and certain soft tissues remained between 4 and 7 %.

On the other hand drying at lower temperatures reduces volatilization losses but exposes the sample to the ambient environment for longer periods of time, thus increasing the risk of contamination. In addition, it is difficult to dry bigger samples satisfactorily.

Regarding the recovery of an element during drying and ashing it has been questioned whether inorganic radiotracers added to biological samples provide an accurate measure of the organically bound elements in that sample (14, 62). A few investigators have also examined the loss of trace elements in certain selected biological



Elements →	K	P	Cl	Na	Fe	Zn	Rb	Mn	Se	Co	Ag
Concentrations* Dry weight	11 · 034 mg/g	6 · 793 mg/g	4 · 143 mg/g	2 · 196 mg/g	936 µg/g	132 µg/g	18 · 20 µg/g	11 · 58 µg/g	1 · 51 µg/g	495 ng/g	15 · 63 ng/g

*) Concentration level found in the normal cattle liver used in this experiment (6 determinations).

Figure 2. A Natural distribution profile of some elements in the cattle liver prior to homogenisation

B Distribution profile of the various elements in the cattle liver following homogenisation, using the Mikrodismembrator

(Iyengar and Kasperek 1977)

samples during drying as well as dry ashing (59, 75, 91). The effects of drying temperature on different biological matrices such as oyster, rat tissues and human urine for the elements Cd, Co, Cr, Fe, Hg, I, Mn, Pb, Sb, and Se are summarized in Table 2. In an animal experiment, following long time incorporation of the radioisotopes of Zn, Co, Se, Sb. I and Hg drying up to 120°C showed no loss of Zn and Co from rat tissues. However, the volatile elements Se, Sb, I and Hg showed tissue specific differences in retention (70, 73). Cr is also a disputed element in this respect and problems concerning determination of Cr in biological materials have been exclusively discussed (6, 15, 75, 82, 85, 103, 136, 137).

Regarding the retention of *selenium* in different biological matrices, it was reported to be lost from milk and egg powder upon oven drying but not on freeze-drying (131). In plant material oven-drying for 6 hours at 100°C resulted in a loss of 2 %, 18 hours of drying under the same conditions resulted in a loss of 6 %, whereas in herbage no loss of Se was reported even at 110°C (103). In an animal experiment, Se volatilization in the rat could be controlled by a proper diet (46). Serious loss of Se to as much as 75 to 84 % was also observed during wet ashing of urine samples, and for this reason a closed system digestion was recommended as the only solution for the accurate analysis of urinary Se (116).

Regarding *mercury* which is labile upon exposure to heat, the extent of loss during oven drying reported in the literature for various biological matrices is very variable. However, there is some consistency in the reported data regarding its loss from fish by both freeze and oven drying procedures (74, 88, 107). In an animal experiment, for brain samples which were spiked with Hg, no loss was reported on drying (44). Whereas a serious loss up to 56.5% in some parts of the brain was reported from another laboratory (107).

Especially for Hg, there are various ways through which it can be lost from sample media which has been the subject of discussion in the following reports (11, 24, 28, 35, 43, 44, 51, 74, 95, 109, 132, 139).

Freeze dryring is also known as lyophilization or vacuum freeze drying. Basically it involves 3 stages, pre-freezing, primary drying and secondary drying. Ensuring adequate vacuum and pre-freezing of the samples prior to lyophilization are the key factors in obtaining good retention yields and prevention of contamination. Failure of vacuum is often reponsible for melting of the frozen sample, promoting the sample-container interactions and the advantage of the frozen state is lost.

Generally freeze drying of biological samples has been reported to be satisfactory for many elements in different matrices such as Se in milk (123), Hg in guinea pig and rat tissues (86), Fe, Cr, Se, Mn and Zn in Oyster (43) and Zn, Co, Se, I, Sb and Hg in a number of rat tissues and human urine (70, 73). However, loss has been reported for Hg in fish (74, 88, 107) and pons, a sub-part of brain (107). In another experiment freeze drying was found to be satisfactory for water samples with respect to a number of elements including Se but not for Hg and I (56).

Table 3 shows the retention yields for various elements in different matrices following freeze drying. Basically freeze drying is well suited for drying biological samples, yet care should be taken to prevent the contamination from the metallic housing of the freeze drier for elements such as Cr, which may volatalise and get entrained in the sample. Use of nonmetallic components such as perspex or preferably quartz is desirable to construct the sample housing compartment of the freeze drier.

3.5 Liquid samples

Liquid biological samples should be generally considered to be heterogeneous. In most cases they are suspensions or emulsions. Therefore it is necessary to decide whether the analysis should be carried out for the total fluid or for the separated components.

3.5.1 Homogenisation

Homogenisation of the suspension or emulsion is rather necessary. This can be done by agitation, stirring, shaking or boiling; squashing, blending and ultrasonic treatment. Aliquoting should be done immediately after homogenisation to prevent segregation. If the fluid is dried at this stage itself, homogenisation of the dry residue is necessary. Homogeneity tests, as discussed under "solids and liquid phase" may be applied.

3.5.2 Phase separation

Phase separation can be carried out without or with chemical change of the phases.

Phase separation without chemical change includes sedimentation, decantation and manual sorting, normal and ultra filtration, normal, differential and ultra centrifugation, subcellular fractionation in order to separate nucleus from the cytoplasm, cell electrophoresis and dialysis.

Phase separation with chemical change includes chemical decay, such as hydrolysis, oxidation, reduction, hemolysis, denaturation of proteins and fermentation. Finally change in the chemical form of an element can alter the behaviour of the phases during separation.

3.5.3 Preconcentration of liquid phase

After phase separation preconcentration of the liquid part may be necessary for subsequent instrumental analysis without further chemical handling. Methods

Table 2:
Loss of elements during oven drying of biological samples

Element	Matrix	Procedure or Mode of Incorporation	Temperature (°C)	Time (h)	Loss observed (%)
Cd	Oyster	radioisotope, metabolized	120	48	no loss
			90	48	no loss
			50	48	no loss
	Rat liver	radioisotope	110	16	1
	kidney	intravenous	110	16	1
Co	Oyster	radioisotope, metabolized	120	48	no loss
	Mollusc	radioisotope metabolized	110	?	14
	Rat, many tissues	radioisotope, intravenous	80	72	no loss
			110	24	no loss
			120	24	no loss
Cr	Rat liver	radioisotope, intravenous	120	48	no loss
	blood		110	16	3
Fe	Oyster	radioactive, intravenous	110	16	5
	Rat blood		105	48	no loss
Hg	Human Urine	^{203}Hg-organic, intravenous	80	72	3
			105	24	15
			120	24	25
	Plankton	chemical analysis	60	50	51–60
	Rat liver	radioisotope, metabolized	80	72	5
			105	24	3–10
			120	24	7–15
	Rat brain	radioisotope, metabolized	120	24	5–16
	muscle		120	24	5–21
I	Human Urine	radioisotope, metabolized	80	72	2
			105	24	4
			120	24	7
	Rat muscle[a)]	radioisotope, metabolized	120	24	< 5
	blood		120	24	7
	serum		120	24	7
	erythrocytes		120	24	8
	brain		120	24	10
	kidney		120	24	15
	lung		120	24	7
Mn	Oyster	radioisotope, metabolized	50–120	48	no loss
	Mollusc		110	?	14
Pb	Oyster	radioisotope, metabolized	60	48	10
			100	48	17
			120	48	20
Sb	Rat blood	radioisotope, metabolized	105	24	< 5
			120	24	< 5
	Rat brain		120	24	8
	kidney		120	24	9
	lung		120	24	6
	spleen		120	24	7
Se	Herbage	chemical analysis	30	12	no loss
			60	12	no loss
			100	12	no loss
	Rat blood	radioisotope, metabolized	120	24	< 5
	brain		120	24	< 5
	lung		120	24	< 5
	muscle		120	24	< 5
	Human urine	^{75}Se organic, intravenous	80	72	12–30
			105	24	30–50
			120	24	50–65
	Oyster	radioisotope, metabolized	60	48	< 5
			100	48	< 5
			120	48	> 20
Zn	Rat blood	radioisotope, intravenous	110	16	no loss
	liver		110	16	no loss
	Rat many tissues	radioisotope, metabolized	80	72	no loss
			110	24	no loss
			120	24	no loss
	Mollusc	radioisotope, metabolized	110	?	9

a) No loss observed for any of these rat tissues below 105°C

Data pooled from (29, 43, 70, 107)

Table 3:
Loss of elements during freeze drying of biological samples

Element	Matrix	Procedure or mode of incorporation	Pressure (Torr)	Time (h)	Loss observed[a] (%)
Co	Oyster	radioisotope, metabolized	?	24	no loss
Cr	Oyster		?	24	no loss
Fe	Oyster		?	24	no loss
Hg	Fish	chemical analysis	?	?	20
	Fish homogenate	chemical analysis	?	?	16–39
		radioisotope, spiking	?	?	no loss
	Butterfish	chemical analysis	?	?	70
	Human brain (Pons)		?	?	18–57
	Plankton		?	?	50–64
	Guinea pig, Rat:	methylmercury (^{203}Hg)	?	?	3
	muscle		0.05	24	3,3
	liver				1,7
	kidney				no loss
	heart				1,5
	blood				2,8
	feces				no loss
	muscle	Phenylmercury (^{203}Hg)	0.05	24	no loss
	liver				2
	kidney				no loss
	blood				no loss
	feces				9,3
	Seacucumber	chemical analysis	?	?	59
	Water		0.01–0.05	48–72	39
	Human urine	^{203}Hg-organic, intravenous	0.05	48	2
I	Water	chemical analysis	0.01–0.05	48–72	32
	Human urine	radioisotope, metabolized	0.05	48	2
Mn	Oyster	radioisotope, metabolized	?	24	no loss
Pb			?	24	no loss
Se	Human urine	^{75}Se-organic, intravenous	0.05	48	3

a) No loss was observed for the elements Co, Zn, Sb, I, Hg and Se for a number of tissues from rat (70)

Data pooled from (43, 56, 70, 74, 86, 88, 107)

generally used include partial or complete evaporation, desiccation, freeze, vacuum or oven drying, complete or fractional distillation, dry or wet ashing after evaporation and protein precipitation.

3.5.4 Solid phase

The solid phase obtained by phase separation can be treated according to the details discussed for solid samples in section 4.

3.5.5 Homogenisation of the liquid

If the liquid is not homogeneous for some reason e.g. turbid appearance and freshly thawed fluids, rigorous stirring or shaking followed by immediate aliquoting is generally satisfactory.

3.5.6 Homogeneity test for the liquid

Pure liquid phases generally do not require any homogeneity tests. Wherever necessary, random subsampling with the help of pipettes and quantitative chemical analysis for elements at different concentration levels should be carried out. The details are discussed under solid samples.

3.5.7 Subsampling

In the case of liquids subsampling is relatively easy. It can be done by measuring either the volume (by pipette, dispenser) or the weight (balance).

3.5.8 Preparation of liquid samples for instrumental methods

Preparation of the liquid for instrumental measurements without further chemical treatment includes evaporation of the solvent, drying of the residue and ashing if necessary. For body fluids evaporation of the solvent and drying of the residue results in a concentration factor of 5 for whole blood, about 12 for blood serum and 30 for urine.

3.6. Preservation and final storage

The need for preservation of the sample comes into picture only when the subsequent analysis is not done immediately following the sample preparation. Common methods used for preservation against microbial attack are drying, refrigeration and deep freezing, addition of preservatives, sterilization, e.g. by gamma rays and use of an inert atmosphere. Various aspects of long and short time preservation of biological samples have been discussed under sampling chapter.

For final preservation, samples in the form of dry powders (preferably freeze dried) sterilized by gamma ray treatment and contained in polyethylene bottles with tight caps extend the shelf life over a few years. Details have been discussed extensively under sampling chapter.

3.7. Sample preparation methods and some special considerations

To a large extent, the preparation of samples for analysis is dependent upon the analytical technique, the nature of the biological matrix, the bulk element composition, the element to be determined and the concentration level. Various aspects concerning the principles, demands, shortcomings and sample handling tools are discussed in the earlier sections. In this section, a few general considerations for preparing the samples for activation analysis and atomic absorption spectrometry and certain methods used by various investigators to prepare certain tissues and fluids for analysis, are summarized.

Sample preparation for *neutron activation analysis*, involves the following difficulties in addition to the precautions against the basic hazards, homogenisation, preconcentration and subsampling.

First of all it is necessary to enclose the samples in high purity and especially cleaned irradiation containers (usually polyethylene or quartz) and care should be taken that the samples are not heated during the sampling process. For long time irradiations in a reactor, unless special cooling facility is available, irradiation of liquids and wet tissues is difficult because of radiolysis. Irradiation of such samples under frozen state minimisezs the container-sample interactions. Various aspects and applications of low temperature irradiation and freezing techniques have been discussed (23, 24, 25).

It is advisable to open the long time irradiated samples under liquid nitrogen cooling. This procedure, in addition to reducing the pressure inside the quartz container also helps to solidify liquid samples for easy handling while breaking the quartz tube. Regarding tissue samples opened immediately after cooling in liquid nitrogen care should be taken to prevent the weight gain by the sample due to condensation of the moisture on the surface of the cooled sample material, if the sample has not been weighed already.

When irradiating fine powders such as milk powder and homogenised soft tissues in quartz tubes, compressing the sample powder into a pellet at the bottom of the tube with the help of a high purity quartz rod of appropriate diameter prevents dispersion of the powder inside the container during irradiation, and facilitates quantitative and easy recovery of the sample material from the broken ampoule. For samples prepared in polyethylene

bags and especially for liquid samples squeezing the air out of the bag just before sealing prevents excess pressure build up during irradiation.

Because of the freedom from contamination after the irradiation, it is a general practice to handle the once irradiated samples with relatively less care. Since instrumental activation analysis permits repetition of the analysis or determining additional elements by reactivation, in order to retain the integrity of the sample, as a general rule only plastic tools should be used to manipulate the sample at all stages.

Sample preparation *for atomic absorption spectrometry.* It involves a number of approaches, since it is a single element technique. For elements such as Cu and Zn in serum a simple dilution with high purity water is often sufficient. Whereas for elements such as As, Ge and Cr pretreatments such as dissolution and extraction are necessary either for preconcentration or for eliminating the matrix effects. All such steps introduce the problem of contamination and reagent blank. For solid samples dissolution of the matrix is almost a must and demands very clean working environment, high purity reagents and skill on the part of the analyst. For example, detailed working procedure and the necessary precautions for the determination by atomic absorption spectrometry has been described for As in blood, feces, urine, hair and nail, Pb in blood, Ni in whole blood and urine and Cd in serum and urine (3).

3.7.1 Hard tissues

Calcified tissues such as bone and tooth present formidable difficulties, especially when homogenisation in the natural state is desired. This difficulty is exemplified by the absence of a standard reference material for bone in its natural form that can be prepared with minimal external contamination. Another difficulty is the variation in the mass of marrow in different types and parts of bone which contribute to the wide dispersion of the results reported in the literature (72).

Big samples of bone can be divided into small pieces by cooling in liquid nitrogen, wrapping in PVC sheets and then fracturing with the help of a nylon hammer. The conventional method of powdering bone using agate, ceramic, tungsten carbide and steel mortors is unsuitable for trace element analysis. Both wet digestion and dry ashing at high temperature have only limited use for calcified tissues for various .reasons (62, 81). Low temperature dry ashing (100–150°C) which has been used by some investigators (21, 77), has been more attractive for its similicity and wider applicability for the non-volatile elements. Concerning the loss of volatile elements, the assessment is more complicated by the unknown biochemical binding of the trace elements in the bone, which is a unique biological material. Use of laser in preparing small portions of bone for chemical analysis has also been reported (63) and deserves to be explored further.

In a recent attempt, brittle fracture technique has been used to pulverize small pieces of bone at liquid nitrogen temperature, using a teflon covered metal ball, teflon vessel and a microdismembrator (68). Due to some technical difficulties use of teflon covered metal balls was not entirely successful, and had to be replaced by pure titanium balls. However, using this method only 2–3 g of bone and single tooth samples can be homogenised.

Because of the above mentioned difficulties, the problem of homogenising bone is still an unsolved one.

3.7.2 Semi hard tissues.

Hair and nail are the two important tissues in this group. Because of the number of difficulties for hair samples as discussed under sampling chapter, preparation of hair samples for elemental analysis is one of the major problems encountered by an analyst and there are disputed claims on the merits of the available washing procedures (9, 10, 12, 28, 31, 45, 48, 57, 61, 80, 104, 115).

Washing procedures. The merits and demerits of the various washing procedures for hair have been extensively evaluated by some investigators (9, 10, 12, 28, 61). All these procedures practically fall into the following major categories: Acetone-ether-detergent wash, hexane-ethanol wash, detergent wash and exclusive EDTA wash. These procedures are aimed to remove oil, dust and other surface contaminants.

Acetone-ether-detergent wash. Hair samples are soaked in acetone for 10 minutes with periodic shaking. Acetone is decanted and the hair sample is soaked in diethyl ether for 10 minutes with periodic shaking. Thereafter, ether is replaced with 10 g per litre solution of sodium lauryl sulphate in a water bath at 30°C for 10 minutes. Finally the hair samples are rinsed 3 times with deionized water and once with acetone and dried at 60°C for 24 hours.

Hexane-ethanol wash. Hair samples are soaked in hexane for 30 minutes with periodic shaking. This solution is decanted and the hair samples are soaked in ethanol water (95/5 by vol) solution for 30 minutes with periodic shaking. The ethanol wash is repeated 3 times. Finally the hair samples are soaked in deionized water for 30 minutes, rinsed 3 times and dried at 60°C for 24 hours.

Detergent wash. Hair samples are soaked in 10 g/l solution of sodium lauryl sulphate for 30 minutes with periodic shaking. After decanting a second wash is given. Following this, hair samples are rinsed 3 times with deionized water for 30 minutes each and then dried at 60°C for 24 hour.

Exclusive EDTA wash. This is done by simple soaking of hair samples in 10 per cent solution of EDTA with occassional stirring, followed by rinsing with deionized water.

Alternatively, the samples are first soaked in deionized water to separate heavier particulate matter trapped in the hair strands. The drained hair is then placed in a detergent solution (e.g. commercial shampoo) and heated to incipient boiling, drained and rinsed with deionized water. The detergent treatment is repeated for a second time, followed by rinsing 2 times with deionized water. This is followed by washing with acetone, drained and placed in a beaker containing saturated EDTA to stand for 5 minutes. After draining the EDTA solution the sample is rinsed with deionized water. The EDTA sequence is repeated once more before finally rinsing twice with deionized water and the sample is dried over night at room temperature.

Effect of washing procedures. The Snoop wash (a commercial detergent), organic wash or the boiling water wash effected no changes in Cu, Zn, Ca or Mg values and Zn was unchanged after most washing procedures excepting chelating procedures (61). EDTA procedure was found to be well suited to eliminate the surface lead contamination of hair (28). Washing with hexane-ethanol or detergent washing of hair samples was found to be satisfactory for Cr (52). In another study a nonionic detergent was recommended to be best for a number of elements (12). Greatest variability was reported for Cu after washing by different procedures (9).

Preparation of nail samples. A variety of methods using detergents or organic solvents used by various investigators have been briefly reviewed (58). Samples treated with nail paints require drastic cleaning procedures to remove the surface contamination completely with the risk of affecting the integrity of the sample. In a recent investigation on samples from Egypt, very high values for Au were observed in such samples (76).

Nail samples are usually obtained with the help of stainless steel scissors or nail clippers. Visible dirt should be scraped using a quartz knife or platinum coated razor blades, and washed several times with acetone and deionized water to remove fat, sweat and dirt and dried at 115°C for 1.5 hr in a closed atmosphere (141, 142).

Soaking the nail samples in 95 % ethyl alcohol for 20 minutes following the scraping of the dirt with the help of stainless steel blades has also been used (93).

In another method, the nail samples are scraped with teflon quoted forceps to remove surface contamination. The samples are soaked in 25 ml of 1 % solution of 7x-o-matic (a non-ionic detergent) and shaken for 30 minutes. The samples are then thoroughly rinsed many times with deionized water and dried over night at 105°C (58).

Conclusion. It is difficult to conclude which of the washing procedures is best suited for washing hair and nail during the sample preparation step. However it may be remarked that chelating procedures generally tend to remove more than the surface contamination and the detergent washes are more advantageous since they correspond closely to in situ washing (61).

3.7.3 Soft tissues

Due to lack of recommended standard procedures for handling biological samples for trace element analysis as yet there is no unified approach for tissue handling. Of basic importance is to keep the tissue frozen solid in order to minimize contamination and preserve the biological integrity. However, instead of fresh tissue, if frozen tissue is handled with stainless steel knife and scalpel because of the greater friction in cutting the frozen solid, contamination from elements such as Cr, Mn, Ni and Co should be anticipated. For example, Cr was found to be increased by a factor more than 10 in an experiment designed to assess the sample handling procedures using boron nitride knives and stainless steel scalpels under different conditions, and it was concluded that no one method was entirely satisfactory (94).

A primary difficulty faced while preparing the tissue for the analysis results from the interfering components such as the connective tissues, capsules, skin, visible fat, blood vessels, nerves, hair (during skin sampling), marrow (during bone sampling), glandular parts, GI-tract contents such as food rests and feces, residual blood, extracellular fluid, trapped plasma or serum in erythrocytes, platelets and leucocytes, erythrocytes and leucocytes in platelet samples which are intricately mixed with the sample material to be prepared for the analysis and are difficult to remove completely. This has been discussed for a few organs such as kidney (89), blood vessels (134), brain (64) and liver (69, 71).

Blood rich organs such as liver, heart, spleen, kidney and placenta present more difficulties compared with other soft tissues. It is not possible to remove the detrital blood completely without damaging in some way the originality of the parent tissue. Invariably this leads to wide spread variation at least in the Fe values, as observed for these tissues (72).

Autopsy samples provide better opportunities for sample preparation since sufficient quantitity of the sample material can be collected by the pathologist. The samples provided by the pathologist should not be penetrated with any of the instruments. They should be enclosed in polyethylene vials and frozen immediately. After shaving off the outer layer with appropriate instruments subsampling may be done either in the frozen state or after thawing, depending upon the organ, if wet random samples are required. However random sub-

sampling in the frozen state does not permit the removal of the interfering components, especially blood from blood rich organs.

In order to remove the interfering components and homogenize the sample, thawing of the sample is necessary. This permits at least partial elimination of blood with a quick distilled water wash. These operations cause the loss of tissue fluids and possible cell damage, which can affect the elemental distribution within the tissue. This remains to be further investigated. Perfusion with isotonic solutions will remove the extracellular fluids and prevents cell damage, but introduces contamination problems.

Biopsy samples can be obtained from a number of organs and tissues such as liver, kidney, muscle, prostate, skin, tooth and bone. Only a few milligrammes of the sample material can be obtained by needle *biopsies*. One problem with such small samples is the difficulty to remove fat, blood and the connective tissues to avoid variation in the results. For example about 1 % of the blood was found in muscle samples (16). Another difficulty is to determine the exact wet weight of the sample if the information is needed on the fresh weight basis. This is generally done by weighing the samples several times at short known intervals of time soon after removal and extrapolating to zero time. Following reports have explored the diagnostic utility of muscle biopsy elemental analysis and the associated difficulties (13, 16, 40).

Problems during the sample preparation of certain typical samples are discussed below.

3.7.3.1 *Liver.* The main difficulty in preparing liver is the removal of the detrital blood which is about 5 per cent by weight, blood vessels and fat. The magnitude of the error in the mean composition of the liver portion analysed depends on the efficiency of the tissue handling since blood (19), blood vessels (134) and fat (118) have different elemental composition.

In the absence of recommended standard procedures liver is handled in different ways by various investigators (69, 87, 94, 117). For example in an experiment designed to study the natural variation of the distribution of 11 elements in bovine liver, the tissue was handled as described below.

About 1 kg of fresh bovine liver was obtained from a butcher who was instructed to cut the liver at one side only, wrap it up in clean PE-sheets and then to freeze it immediately after removal. Further treatment involved cutting off 1 to 2 cm around the edge of the liver. Plastic forceps and an especially prepared suprasil quality quartz knife were used throughout. The liver was cut into 3 big slices to free the tissue from most of the visible non-liver components, such as fat and blood vessels. The slices were then rinsed 3 times in quick succession with penta-distilled water and gently squeezed inside clean PE-sheets between the rinsing operations. Thus it was possible to eliminate most of the blood. Subsequently the liver slices were freeze dried and wrapped separately in several folds of clean PE-sheets. After cooling in liquid nitrogen they were gently pressed between two perspex plates. This treatment subdivided them into small pieces from which most of the remaining smaller blood vessels could be removed. From these smaller blocks few pieces were retained as random samples. The rest which amounted to about 120 g of dry tissue was homogenised and stored in clean PE-bottles. However, this method does not eliminate the extracellular fluid completely and there is also the risk of damaging some cells while washing with water, since it is not isotonic (69).

3.7.3.2 *Kidney.* The presence of blood presents some difficulties in separating medulla from the cortex. However, if the sample is freeze dried prior to sample preparation the two components are easily separable and the capsule can also be detached readily (54).

3.7.3.3 *Brain and lung.* Since these two are symmetrically developed organs duplicate sample preparation is possible. Such samples not only provide the elemental composition profile in functionally same areas but also serve as controls to the analytical method used. Brain has several sub-parts which vary in their trace element composition (64).

Regarding lung, close physical examination of the inside wall of the sample is recommended during sample preparation in order to check the depositions of dust particles which are not cleared by the circulating blood, especially in samples from subjects living in highly polluted/dusty regions for long periods of time.

3.7.3.4 *Placenta.* Placenta is a difficult sample to handle. It consists of mostly clotted maternal and fetal blood and decidual tissue contaminated with amniotic fluid. Since placental samples are usually received from the maternity ward in the frozen state, prior to sample preparation thawing is necessary in order to eliminate the trapped fluids. Using a perforated hollow cylinder and a suitable piston made of teflon part of the fluids from the thawed samples can be gently forced out. However, excess squeezing and washing with water to achieve maximum elimination should be avoided. Homogenisation is very important and it is advisable to homogenise after drying.

3.7.4 Biological fluids

Biological fluids are more susceptible to bacterial growth in unfrozen state and sample preparation should be finished preferably within 24 hours. Among the vari-

ous biological fluids whole blood, serum, plasma, urine, milk and cerebrospinal fluid are the easily accessible common specimens. Other less common specimens in this group include semen, tears, sweat, sputum and saliva. Samples from fluids such as synovial, amniotic, pancreatic, prostatic, bile and gastric juice require special procedures. Some aspects of specimen collection, preparation and stability of biological fluids with respect to trace element analysis have been excellently reviewed (5).

Procedural details concerning a few typical biological fluids are outlined below.

3.7.4.1 *Blood.* Blood is conveniently obtained by venous puncture. Since various considerations limit the wide spread use of optimal needles made of alloys such as Pt-Rh for drawing blood, disposable type of needles and syringes are generally used. Keeping all the practical limitations in mind, collection of blood in 10 or 20 ml fractions successively using the same needle, and discarding the first two or three as rinse fractions appears to be a practical solution. However, this method is not entirely suitable for elements such as Cr and Mn. On the other hand, use of plastic catheter reduces the contamination several folds for the difficult elements such as Mn (122), as well as the discomfort to the subjects since a single rinse provides a valid sample.

Neonatal blood samples. In addition to the difficulties listed above, preparing neonatal blood samples present additional problems since only a small volume of the sample can be obtained because of certain pediatric considerations. A technique of handling these samples using a flexible teflon tubing to sample capilary blood from the heel for elemental analysis has been discribed (97).

Hemolysis. It is important to avoid hemolysis, since elements such as K and Fe, which are at a higher concentration in erythrocytes, may affect the serum value depending upon the degree of hemolysis. Visible hemolysis begins at about 50 mg of erythrocytes per 100 ml serum. Use of a dry syringe, slow transfer to a dry test tube and sufficient time for clotting is necessary. Fresh blood, blood from new born and blood which has not sufficiently clotted increase the risk of hemolysis. Upto 0.3 % hemolysis does not affect the Cu and Zn values in serum (90). However in pediatric serum samples high value for Zn following hemolysis has been reported (92). Further discussions on the effect of hemolysis may be found in the following reports (34, 66, 110).

3.7.4.2 *Serum.* As a rule of thumb serum should be separated from the clot within one hour (4). The clotting of blood takes about 15 minutes at room temperature and will be delayed when siliconized glassware, teflon or PE-containers are used. Fractionation of blood by centrifugation is done at about 3000 rpm for 10 to 15 minutes. The contents of the tubes should be kept closed until separation to prevent contamination and loss by evaporation. Recentrifugation of the separated serum is sometimes necessary to spin down the residual erythrocytes. One disadvantage with serum is that hemolysis is greater than in plasma and clotting releases K from platelets. For this reason, normal K values are lower in plasma than in serum (72). Zn may also be affected in the same way (42).

3.7.4.3 *Plasma.* The main difference between serum and plasma is the presence of fibrinogen in the latter. Plasma can be obtained by immediate centrifugation of heparinized blood, but the presence of the anticoagulant is undesirable for trace element analysis. However, it is possible to obtain plasma without an anticoagulant if fresh blood is immediately centrifuged (2). The conditions are same as described under serum.

3.7.4.4 *Erythrocytes.* Careful removal of all the serum is necessary to separate the erythrocytes. However, a certain amount of trapped serum (usually 5 to 8 per cent) is unavoidable and needs correction. Check on hematocrit is recommended if erythrocyte values are used to compute whole blood values and viceversa.

3.7.4.5 *Urine.* Problems concerning trace element analysis of urine have been thoroughly assessed with respect to contamination, sampling and sample preparation (32). A few practical hints are outlined below to obtain 24 hour collections, as random samples have only limited qualitative use.

24 hour collections are made after omitting the first voiding at the start and including the last one at the end of 24 hours into high pressure PE-bottles, pre-cleaned by leaching separately with HNO_3 and H_2O_2 for a few hours followed by liberal rinsing with double distilled water. Following the sample collection aliquoting is done as soon as possible after vigorous shaking of the contents using pipettes which are rinsed several times with the urine to be transferred. It is preferable to freeze and lyophilize these subsamples (15 to 10 ml) to avoid the interaction of urine with the container. It should be noted that some patients wear metal catheters in the urinary passage which might contaminate the samples if obtained from them. Collection in pyrex bottles in troduces serious contamination for Cu (27).

Drying of urine. Oven drying of urine is unsuitable for trace element analysis as the matrix begins to decompose even at 80°C resulting in the loss of residual dry matter. In an experiment with human urine severe loss of volatile elements especially of Hg and Se upto 15 and 30 % respectively was observed on oven-drying at 80 and 105°C. However no loss was observed on freeze

drying (70). But freeze drying has the following disadvantages. It is not practical if the number of samples are too many and the sample size is large. Freeze dried urine residue is hygroscopic and additional precautions are necessary to prevent this.

For these reasons, wherever the technique permits, elemental analysis on whole urine is recommended.

3.7.4.6 *Milk.* Recently analysis of human milk has gained considerable importance in relation to infant nutrition. Samples of breast milk are collected after cleaning the nipples and wiping them dry with cleanex, either by manual expression or by using plastic breast pumps. It is necessary to empty at least one breast completely and mix before collecting the sample as the mean composition changes during the feed itself e.g. fat content. Status of the sample i.e. colostrum, transitional or mature should be clearly defined since stage of lactation influences the protein content and therefore the metal concentration.

3.7.4.7 *Sweat.* Sweat is usually collected either by the so called arm bag method using a PE-bag around the arm or from the whole body by collecting the free flowing drops from various points of the body. This is done in specially created sweating environment after the subjects have showered. However, collection of a valid sweat sample is difficult because of the numerous contamination hazards. The sweat collected should be homogenised by rigorous shaking and prepared for the analysis immediately. It is also necessary to centrifuge in order to obtain cell free sweat. In addition, unpredictable dilutions may occur since the profuseness of sweating greatly varies in different parts of the body (31, 65, 126).

3.7.4.8 *Excreta.* Like urine only timed collections up to 48 or 72 hours are useful for metabolic studies. This creates unpleasant handling problems since it is necessary to homogenise the bulk before proportionating for analysis. Freeze drying is by far the best method since it also deodorizes to a great extent. Contamination from urine and blood, especially when samples are collected from menstruating females, should be checked. Marking feces with different colouring agents to detect the beginning and end of a collection period may be used.

3.8 Literature

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4. ASHING (in cooperation with V.K. PANDAY)

4.1 Introduction

Trace and ultratrace element analysis of biological material is in general disturbed by a million to billion fold excess of organic compounds, which represent the matrix (116, 117, 130). Therefore, complete elimination of all organic matter is often unavoidable before the subsequent steps of dissolution, preconcentration, separation and preparation of the measurement sample. Ashing can also be used as a tool for preconcentration in case of pure instrumental trace element analysis with concentration factors of up to hundred fold for soft tissues and still more for body fluids.

The organic matrix can, in principle, be eliminated either by extracting the trace metals from the matrix or volatilising the matrix by ashing. Extraction, however, is limited only to a few cases, where the trace metals can be extracted quantitatively and where the matrix is completely insoluble. Ashing, thus is an important step in trace element analysis.

Ashing may be defined as the removal of organic matrix by converting it into suitable gaseous components which are then volatilised, leaving behind an inorganic residue from the original biological material for element analysis.

An ashing method to be used for trace element analysis shall ideally involve:

a) complete removal of all organic components of the sample;

b) retention of all elements to be determined quantitatively within the inorganic residue; and

c) avoid all possible contamination, external or otherwise.

The (a) complete removal of organic matter is desirable not only for separation and preconcentration steps but also helps guard against uncontrolled and interfering complex formation of the analyte with the remaining organic residue. The extent of interference however, varies widely with the method of measurement to be employed. To cite an example, voltammetry is especially sensitive against interfering traces of organic matter. Atomic absorption and flame photometric methods have been widely employed without ashing the materials completely (26). Activation analysis and x-ray fluorescence analysis under favourable conditions can be virtually non-destructive (46) in several cases, if preconcentration is not resorted to.

With respect to (b) ashing operations are always subject to loss of several trace elements by volatilisation (25, 28, 31, 50, 63, 89, 102) and/or adsorption (108, 122). The verification of c) which may occur either from the apparatus or the reagents used, has to be made carefully through blank determinations.

In its chemical principle, ashing uses generally a powerful and quantitative oxidation of the organic material into gaseous oxidation products such as H_2O, CO, CO_2, SO_x, N_2 which are subsequently volatilised (17). Only in a few cases reduction to H_2O, CH_4, NH_3, H_2S, PH_3, HF, HCl, HBr, HI, AsH_3, Hg, Zn, Cd has also been employed. The ashing methods have invariably been categorised under dry or wet oxidation methods (17, 46), depending upon the application of a gaseous or liquid reagent. It is possible to subdivide them further into lower temperature and higher temperature ashing methods according to the temperature employed, high or normal pressure methods, according to the pressure used. The main reaction mechanism of dry ashing at high temperatures in the muffle furnace is air oxidation in combination with pyrolysis. Wet ashing at higher temperatures with mineral acids as oxidants involves oxidation together with dehydration.

Bock (17) and Gorsuch (46) give various reagents which although do not lead to complete destruction of organic matter, are at the same time suitable for conversion of the analyte into simple ionic species amenable to the analytical measurement. Sometimes the breakdown of groups which complex the analyte may be sufficient e.g. use of sulphuric acid and permanganate for preparation of samples prior to mercury estimation (7, 92). The ashing in the graphite tube of a flameless atomic absorption spectrometer is also some type of only cracking and not ashing the organic material and has been attempted as a means of direct introduction of samples without any pretreatment (42, 80).

Without any doubt, however, complete removal of all organic compounds is more safe.

The various methods of ashing are listed in Table 1. Among these, of major importance to trace element analysis in biological materials are dry ashing with air in the open muffle furnace or with oxygen plasma at temperatures below 150°C, oxy-hydrogen combustion at very high termperatures, wet ashing by $HNO_3/HClO_4$ and by radicals from iron(II) catalysed H_2O_2 at temperatures below 110°C. They are treated in the following in some detail.

4.2 Dry ashing

Combustion with air or oxygen has been one of the earliest ashing techniques. Combustion of organic material by oxygen from air in open sytem still was used by

Table 1:
Ashing of organic material for trace element analysis

1. DRY ASHING	2. WET ASHING	3. OTHERS
1.1 Higher temperatures 1.1.1 Combustion with air or oxygen 1.1.1.1 Stationary system 1. Open muffle furnace, air under atomospheric pressure ("dry ashing") - without reagent addition (19, 34, 76, 116) - with reagent addition (30, 62, 104) 2. Oxygen flask, atmospheric pressure (HEMPEL - or SCHÖNIGER flask (10, 52, 81, 103, 114, 115) 3. Oxygen bomb, high pressure (Colorimetric or BERTHELLOT - bomb) (12, 16, 41) 1.1.1.2 Streaming system 1. Oxygen or air stream in combustion tube (49, 105) 2. H_2/O_2-flame in closed cooled system (WICKBOLD-combustion) (105, 37, 75, 112) **1.2 Pyrolytic decomposition** 2.1 Heating under inert gas e.g. Ar, N_2 ("Ashing" in electrothermal graphite tube AAS) thermal cracking (42, 80) 2.2 Reduction with H_2 at high temperature (20, 119, 129)	**2.1 Normal pressure** 2.1.1 Oxidizing mineral acids, concentrated 1. HNO_3 (17) 2. H_2SO_4 (17, 46) 3. $HClO_4$ (3, 13, 17, 18, 46, 109, 110, 111) 4. HNO_3/H_2SO_4 (17, 45, 46) 5. $HNO_3/HClO_4$ (27, 45, 46) 6. $HNO_3/H_2SO_4/HClO_4$ (45, 46) 2.1.2 Hydrogen peroxide 1. 30 % H_2O_2 (17, 27, 60) 2. 50 % H_2O_2/conc. H_2SO_4 (17) **2.2 High pressure** 2.2.1 Oxidizing mineral acids in "teflon-bomb" (1, 2, 11, 41, 104) 1. HNO_3 in quartz tube (CARIUS) 2. HNO_3 in teflon bomb 3. HNO_3/H_2SO_4 bomb 4. HNO_3/HF bomb 2.2.2 Hydrogen peroxide 30 % H_2O_2 in high pressure bomb	**3.1. Oxidation** 3.1.1 Oxidative fusion, e.g. nitrate (9, 21, 33) 3.1.2 Oxidation in nitric acid vapor (118) 3.1.3 Oxidation with ozone (65) 3.1.4 Halogenation (30, 40, 66, 67, 124, 128) **3.2. Reduction** 3.2.1 Reductive decompostion (14, 17, 22, 24, 38, 77, 112, 123)
1.2 Lower temperatures 1.2.1 Plasma of oxygen gas, at 100 to 125 (250)°C 1. Radiofrequency electrical field (43, 44, 78) 2. Microwave electrical field (59)	**2.3 OH radicals from iron(II)-catalysed H_2O_2 at <115°C (FENTON'S reagent)** (69, 72, 96, 97, 98, 99, 100, 101) 1. Open beaker 2. Closed system	3.3 Enzymatic decomposition (51, 68, 82)

B. Sansoni, V.K. Panday, 1978

Lavoisier in 1781 (76). The basic advantages of dry ashing methods consist in freedom from contamination due to any reagent (except oxygen or air), in their ability to ash large sample amounts without much supervision and in routine operation of a large number of samples.

Because of the trace element losses due to volatilisation at higher ashing temperatures (above 500°C), the incomplete or very time consuming ashing at temperatures below 400°C, along with sometimes inconvenient properties of the ashes obtained, use of dry ashing methods in trace and especially ultratrace element analysis has decreased drastically within the last decade. Exceptions are lower temperature ashing with oxygen gas plasma below 150°C, and combustion in oxy-hydrogen flame at extremely high temperatures of about 2500°C, which for otherwise difficult to ash materials are getting increasing attention.

4.2.1 Dry ashing in muffle furnace

In 1845 Erdtmann (34) carried out dry ashing in open air, in a furnace. The usual technique of dry ashing in a stationary system has been described by Thiers (116) and consists of placing the dry organic matter in a muffle furnace which is then heated to a desired temperature over a period of time. The sample is left at this temperature till ashed to a white powder. Sometimes, charred ashes have to be treated afterwards by wet ashing with small amounts of HNO_3 or H_2O_2 in order to get carbon free, white ashes. The operation is carried out generally in 3 or 4 steps (17):

a) drying at 105 - 150°C
b) preashing in a temperature gradient from 200 - 400°C
c) main ashing between 450 - 550°C which may in some cases be raised to 650°C
d) aftertreatment, if necessary, at 700 - 800°C in case of non volatile trace metals or wet oxidation with HNO_3 or H_2O_2.

The pretreatment at low temperatures can be carried out over a flame; in an apparatus of the type recommended by Thiers (116) or in an inert gas atmosphere, to avoid the possibility of ignition and the ability to use fairly high temperatures for achieving rapid thermal degradation of the organic matter. Boppel (19) has reported a muffle furnace dry ashing method at higher temperatures for radioactivity monitoring of large samples up to 1 kg range in reasonably short ashing times.

The main advantages of the muffle furnace ashing are its simplicity and freedom from contamination due to any reagents, except air. It also requires little supervision and is capable of ashing large samples as well as of large sample numbers in routine operation. These are overcompensated, however, by the disadvantage of volatilisation of a large number of elements such as Ag, As, B, Cd, Cr, Cu, Fe, Ga, In, Pb, Sb, Te, Zn, (25, 28, 31, 48, 63), often comparatively long ashing times, loss of ash, retention losses (108, 122) and impurities introduced through the air (116). Moreover, the reaction mechanisms due to local overheating are uncontrolled and may change from oxidation to reduction within different parts of the same sample. Due to addition of fresh air, local overheating within parts of the sample up to 800°C has been reported, while the oven temperature was only 450°C. Table 2 summarises losses reported for some elements in different biological matrices during ashing.

Reduction of some metal ions by carbon at high temperatures may also sometimes cause trouble. The formation of insoluble silicates or phosphates may occur (46, 87). Melting of trace elements into the wall material of the porcelein crucible also may occur at higher temperatures (17). There is also a large burden of penetrating smell in the whole laboratory during dry ashing of large amounts of biological materials (97).

The addition of various reagents to aid the ashing process by facilitating the decomposition, or for reducing volatility losses, has been well known (30, 62, 104). Various specific reagents as catalysts have also been studied (39). But these are potential contaminants in trace analysis and must be added with caution.

It is not surprising that the amount of 49 % classical dry ashing methods reported in literature since the review of Gorsuch (45) because of the main disadvantages such as loss of trace elements due to volatilisation and incomplete ashing within reasonable ashing times has decreased within the last ten years.

4.2.2 Combustion in oxygen flask

Combustion in a closed stationary system has been proposed (52, 103, 114, 115) to reduce impurities from air and the losses of readily volatilised elements such as halogens, sulphur, phosphorus, boron, mercury, arsenic and antimony. To achieve complete combustion of 1 g material, a flask of 10 liter is required which seriously increases the magnitude of adsorption errors (120).Belesli and co-workers (19) used a flow through oxygen flask which permits handling larger samples than is possible with the standard Schöniger technique. Morsches and Tölg (91) have modified the well tried static combustion procedure for small samples, which is commercially available from Fa. Anton Paar K.G., A–8054 Graz, Austria, (Model VAE Trace-O-Mat). Komissarova and co-workers (64) have described the use of an oxygen flask for mineralisation of plant material to determine phosphorus.

The method has been found suitable for many volatile elements eg. mercury, but has not been found adaptable to arsenic, lead and bismuth which react with the platinum sample holder (46).

Table 2:
Loss of elements during dry ashing of biological samples

Element	Matrix	Procedure or Mode of incorporation	Temperature (°C)	Time (h)	Loss observed (%)
Ag	Animal, liver	chemical analysis	450	?	< 5
	kidney		450	?	< 20
Al	Animal, liver	chemical analysis	450	?	16
	kidney	chemical analysis	450	?	12
As	Ox, blood (dry)	radioisotope,	850	16	35
		spiking	550	16	29
			450	16	28
	Rat, bone	radioisotope,	450	16	44
	blood	intravenous	450	16	86
	kidney		450	16	82
Ba	Animal, liver	chemical analysis	450	?	
	kidney		450	?	4
Ca	Human, rib	radioisotope,	420	16	< 1
		spiking	600	16	< 1
			710	16	< 1
Cd	Animal, liver	chemical analysis	450	?	< 0.7
	kidney		450	?	< 6
	Rat, liver	platinum dish,	600	16	1.6
	liver	chemical analysis	500	16	2
	kidney		500	16	4.4
Co	Animal, liver	chemical analysis	450	?	< 14
					14
	Mollusc	radioisotope,	450	?	26
		metabolized	800	?	22
Cr	Sugar, refined	graphite furnace	450	?	0
	brown		450	?	13
	unrefined	chemical analysis	450	?	47
	Mollasses		450	?	52
	Sugar, refined	muffle furnace	450	?	63
	brown	chemical analysis	450	?	62
	Sugar, unrefined		450	?	86
	Mollasses		450	?	89
	Animal, kidney	chemical analysis	450	?	< 25
	liver		450	?	< 7
	Rat, liver	radioisotope,	700	16	2.2
		platinum dish	500	16	6.1
	Rat, blood		700	16	51.3
			500	16	4
Cu	Animal, kidney	chemical analysis	450	?	0.4
	liver		450	?	0.2
Fe	Animal, kidney	chemical analysis	450	?	0.1
	liver		450	?	0.3
	Rat, liver	platinum dish,	500	16	no loss
	blood	chemical analysis	500	16	0.4
Hg	Fish (whole)		110	24	81.4

Table 2:
(contd.)

Element	Matrix	Procedure or Mode of Incorporation	Temperature (°C)	Time (h)	Loss observed (%)
K	Human, rib		420	16	< 1
			600	16	55
			710	16	90
Mn	Mollusc	radioisotope,	450	?	15
	metabolized,		800	?	21
	Animal, kidney	chemical analysis	450	?	0.4
	liver		450	?	0.3
Mo	Animal, kidney	chemical analysis	450	?	< 1.5
	liver		450	?	< 0.4
Na	Human, rib	radioisotope,	420	16	< 3
		spiking	600	16	10
			710	16	20
	Ox, blood		450	16	slight
Ni	Animal, kidney	chemical analysis	450	?	< 15
	liver		450	?	3
Pb	Animal, kidney	chemical analysis	450	?	< 12
	liver		450	?	< 2.4
	Human, rib		600	16	< 5
			710	16	40
Sn	Animal, kidney	chemical analysis	450	?	< 0.3
	liver		450	?	< 11
Sr	Animal, kidney	chemical analysis	450	?	< 0.5
	liver		450	?	< 2.5
	Ox, blood	radioisotope, spiking	450	16	9
	Rat, bone	radioisotope,	450	16	slight
	blood	intravenous	450	16	16
	kidney		450	16	5
Zn	Mollusc	radioisotope,	450	?	33
		metabolized	800	?	44
	Seaweed	chemical analysis	500	16	no loss
			1000	16	no loss
	Mussels	chemical analysis	500	16	no loss
			1000	16	no loss
	Ox, blood	radioisotope,	450	16	no loss
		spiking	550	16	no loss
			850	16	no loss
	Rat, blood	new porcelain, chemical analysis	700	16	1
	Rat, blood	chemical analysis	500	16	no loss
	Animal, kidney	chemical analysis	450	?	< 1
	liver		450	?	< 1
	Rat, liver	etched porcelain	700	16	1.1
		chemical analysis	500	16	1.3

Data pooled from (45, 50, 53, 57, 63, 93, 95, 113, 131)

4.2.3 Combustion in Oxygen bomb

The organic matter can also be burned in a calorimetric bomb filled with oxygen under pressure (12). Bock and Jacob (16) recovered 1 μg of selenium quantitatively after combustion and passing the gaseous products through a suitable absorption system. The method is well suited to various organo-metallic compounds.

Fujiwara and Narasaki (41) also developed a bomb for trace element determination in organic materials.

4.2.4 Combustion in oxygen gas stream

The combustion of organic material in a flowing stream of oxygen has been widely used for volatile elements (49, 105) in different types of combustion tubes. Radmacher and Hoverath (94) have improved the technique greatly by employing the principle of controlled combustion. The method has been applied to different types of materials, but is mainly used for determination of C, H and N in organic substances.

4.2.5 Combustion in oxy-hydrogen flame

Combustion in an oxy-hydrogen flame provides a powerful, complete and rapid method for dry ashing of organic matter, which otherwise is difficult to ash. However, it was not as yet much recognised in biological or environmental trace analysis.

Its principle first described in 1922 by Voigt (126), the method has been developed by Wickbold (112) using an oxy-hydrogen flame within a closed quartz apparatus for determining halogen and sulfur in organic compounds. The organic material to be ashed, primarily as a volatile liquid, is transported as vapor together with oxygen, hydrogen or an inert gas stream into the oxy-hydrogen flame, where it is combusted quickly and completely because of the high temperature of about 2500°C. The combusted vapor passes through a condensing quartz tube into an absorber solution, where the halogens or sulfur can be determined (35).

Ehrenberger, Gorbach and Hommel (37) have modified the apparatus to allow for continuous combustion of a large number of samples for routine analysis. Kunkel (75) modified the burner for viscous liquids. Solid samples have been ashed by Ehrenberger (36) using his cascade burner arrangement. Kunkel (75) also proposed a burner for solid samples, which incorporates a preashing chamber for up to 50 g of material. This equipment is commercialy available in four different versions from Heraeus Quarzschmelze GmbH., Hanau, FRG. The version No. IV is especially suitable to biological and environmental solid material.

The Wickbold combustion has been used for determining trace elements such as Hg (15, 61, 74, 106), As, Cd, Pb, Se, Zn (35, 73, 133) in organic material, plastics, biological and environmental material. Fat can be ashed easily and completely after dissolving in an organic solvent. According to Kunkel (75), an oxy-hydrogen-chlorine flame, produced by adding CCl_4 or HCl to the oxy-hydrogen flame, is advantageous for determining trace metals because of the formation of volatile chlorides. Plastics have also been ashed by this method.

There is no doubt, that the method has the potential of a powerful ashing technique for biological and environmental materials.

4.2.6 Pyrolytic Decomposition

Decomposition under inert gas atmosphere is generally useful during preashing steps and is followed usually by further ashing in presence of oxygen. However, ashing in the graphite tube of an electrothermal atomic absorption spectrometer has been used as a means of direct sample introduction for biological materials (42, 80) as stated before.

Pyrolytic decomposition/reduction in hydrogen atmosphere has been used long ago and is becoming widely used for determining traces of carbon, nitrogen, phosphorus and sulphur (29, 47,83, 120).

4.2.7 Low temperature ashing in oxygen plasma

The ashing of organic matter under the influence of a high frequency/ electromagnetic field first proposed by Lee and Seaton (78) and latter by Gleit and Holland (43, 44) has by now become a promising ashing technique. The oxygen plasma produced by an electric glow discharge from oxygen gas flowing through it under low pressure, contains ions, excited atoms and radicals which have a high oxidising power and are effective in decomposing the organic matter at low temperatures. Commercial instruments utilising this principle are now available and it has been claimed that this approach is superior in that volatile elements are not lost and that ionic material and morphology are retained (88, 125). The temperature in the reaction vessel can be controlled by the power fed into the plasma, which may be around 300 watts.

The name cold ashing normally associated with this is, however, not true in practice, since the temperature range usually is 100 to 125°C and can be raised considerably, up to 150 or even 200°C, depending on the energy applied. The method can be made to work at reasonably low temperatures, resulting however, in long ashing times. It has the advantages of dry ashing techniques viz. no addition of reagents and thus little possibility of contamination, no reagent removal after ashing and no explosion hazards. Although the manufacturers of commercial instrumentation claim some-

times that there are no volatility losses by this approach, losses of elements e.g. As, Au, Cd, Hg, I, Se, have been reported (48, 127). However, suitable traps can be constructed to reduce these losses (120). Equipment is commercially available by the manufacturers of International Plasma Corporation, Tracerlab resp. Trapelo and Erbe Elektromedizin KG, Tübingen (Model 120 MK 2), LFE Process Control Division, Waltham, Mass., USA.

The method is comparatively faster than the muffle furnace technique under comparable conditions (ashing of 25 g rice needs 30 hrs compared with 5 days in a muffle furnace.) In case of 5 g dry blood, however, one of the authors (S.) even after seven days ashing at 1 Torr O_2 pressure and 220 W could not reach constant weight. The ashing times can be shortened by use of an O/F plasma produced in an O_2/CF_4 atmosphere.

The method is most suited for dry ashing of samples with relatively large outer surface, e.g. membrane filters, plant material such as leaves and thinly spread whole blood. For bulky materials, the method sometimes cannot be recommended.

This principle has been further adapted by Kaißer Tschoeppel and Tölg, (59) to the peculiar problems of ultratrace analysis, by achieving excitation of oxygen in a microwave resonant cavity operated at 2450 MHz with variable power upto 200 W. The authors were able to ash practically all organic matrices including plastics, slimes, soot etc. in amounts up to 1 g, and recover nanogram amounts of volatile elements as e.g. Hg in a cold finger trap. The approach should find applications in forensic and medical sciences.

4.3 Wet ashing

Main advantages of wet ashing over dry ashing are the lower temperatures resulting in lower losses of trace elements and faster and often more complete removal of organic substances. Because of this, wet ashing in trace element analysis generally is preferred over dry ashing in a muffle furnace. Losses of element traces due to reaction with container walls and mechanical losses of ash are also smaller.

Disadvantages are the excess of concentrated mineral acids and their subsequent removal, the temperatures of up to 300 to 350°C with losses of some elements. The addition of reagent includes the danger of contamination and higher blank values. Aggresive fuming vapor in case of mineral acids needs special fuming cubs. In this respect H_2O_2, therefore, has advantages over the strong mineral acids.

4.3.1 Higher temperatures, normal pressure

The reagents commonly used for wet ashing at higher temperatures are the strongly oxidising mineral acids HNO_3 (69%), H_2SO_4 (98 %), $HClO_4$ (72 %) and furthermore H_2O_2 (30 %; 50 %) or suitable combinations of these. The higher temperatures are employed to overcome the relatively high activation energies for oxidative breakdown of the chemical -C-C bonds in the organic molecules under study. The mechanism of oxidative breakdown proposed by Shemyakim and Shchukina (107) conforms well with the use of various standard mixtures in wet ashing methods. The use of nitric acid alone is often not sufficient and, is generally made in combination with concentrated sulphuric acid. The action of nitric acid is supposedly via

$$2\ HNO_3 \rightarrow H_2O + 2\ NO_2 + 1/2\ O_2$$

with both aromatic and aliphatic organic material, leading to oxidation, esterification and nitration.

The interaction of **sulphuric acid** with organic compounds is complex, though mainly oxidation together with dehydration (and pyrolysis) probably occur. The presence of H_2SO_4 also helps to raise the boiling point which may be both an advantage as well as a disadvantage. Since concentrated sulphuric acid alone oxidises too slowly, nitric/sulphuric acid mixtures have been the most important classical wet ashing reagents. **Perchloric acid** alone is very powerful ("liquid fire" according to G.F. Smith) and efficient in destroying organic matter. It has a boiling point of 203°C and forms an azeotropic mixture containing 72.4 % perchloric acid at this temperature. The danger from the use of this acid arises due to the formation of various unstable perchlorates. A great deal of work on the use of this acid has been carried out by Smith (109, 110, 111) who has stated that the danger arises with the use of perchloric acid with hydroxyl compounds which form unstable perchlorate esters. Often preoxidation with nitric acid can be employed to remove this danger. Perchloric acid should therefore be used only after dilution with HNO_3, as e.g. 1 Vol. $HClO_4$: 2 Vol. HNO_3 (3,58). Gorsuch (46) states that milk fat was found to be most dangerous of all the fatty material handled by him.

A code of practice for handling perchloric acid has been published (3) and should be studied before use of this acid. These include:

1. Do not bring materials containing hydroxyl groups into contact with perchloric acid. This includes items such as paper tissues which should not be used for mopping up spills etc.
2. When oxidising hydroxylic materials always ensure that adequate preliminary oxidation with nitric acid occurs.
3. Exercise great care when oxidising immiscible materials. Test a small quantity first, but remember that scaling up can introduce extra hazards.
4. If the oxidation mixture chars, a brown colour appears, or if there is any sign of difficulty dilute and cool the mixture at once.
5. There is evidence that continuous use of perchloric acid in wooden framed fume cuppoards can lead to

impregnation of the wood, and an increased explosion hazard. Ceramic fume cupboards, which can be rinsed with water are commercialy available and are to be recommended.

The use of perchloric acid/nitric acid for decomposing various types of biological matter has been reported variously (13, 18, 45, 54, 90). The use of a microwave oven for wet diqestion has been reported.

The pros and cons of the use of perchloric acid are most appropriately summed by Gorsuch (45) when he writes that having carried out hundreds or even thousands of oxidations without incident, but having seen the aftermath of one explosion, I would probably avoid its use, other than in exceptional circumstances.

All this said and done, without doubt today $HNO_3/HClO_4$ belongs to the most powerful of all wet digestion mixtures especially for decomposing otherwise difficult to ash materials, including fatty matter. The resulting perchlorates are easily soluble except $KClO_4$ which may be dissolved in excess of water. Care should be exercised in ashing nitrogen containing heterocyclic compounds which are not easily destroyed and result in loss of trace elements as volatile chlorides.

Hydrogen peroxide has been well known as a powerful oxidant for sample dissolution since the investigations of Classen and Bayer (27) and Kleeman (60). Nevertheless its applications have been mostly in combination with other oxidising agents or for removing traces of colour in charred residues from dry ashing.

The commercial production of 50 % hydrogen peroxide with a high level of purity (100, 101) has resulted in its use for oxidation of biological matter. It is used in conjunction with sulphuric acid which dehydrates the sample to charring before H_2O_2 is added dropwise. The oxidations involve permono sulphuric acid which introduces oxygenated groups into many kinds of organic molecules. A large number of organic material can be ashed, including fat, oil, paraffins and several types of plastics. Graphite and polypropylene cannot be ashed easily, however. Advantages of this method consist in its rapidity and complete ashing. However, though H_2O_2 may be of extreme purity, the purity of sulphuric acid may be a cause for some concern and doubt, in addition to its disadvantages in terms of forming in soluble sulphates eg. Ca, Pb, Ba and the need of subsequent removal of excess H_2SO_4 after ashing.

Chloric acid ($KClO_3/HCLO_4$) has also been described for digesting biological material (5).

4.3.2 Wet ashing in closed systems under pressure

The use of pressure systems has been proposed to diminish volatility losses and decrease in the quantity of reagents. Bernas (11) describes the advantages of closed systems for sample decomposition compared to open systems and concludes that these are superior in terms of many advantages. Wet ashing of biological material with HNO_3, H_2SO_4, $HClO_4$ and HF and corresponding mixtures under high pressure has been gaining popularity (1, 2, 41). The principle of the method is elevation of boiling point with increased pressure.

Care should be taken that safety valve is functioning properly. The use of H_2O_2 and $HClO_4$ (1, 2) has been made with special modifications. Care should be taken when wet ashing fat with HNO_3 under pressure because of possible reactions with the glycerine obtained by fat hydrolysis resulting in formation of nitro glycerines.

Equipment is commercially available from Parr Instrument Company, Moline, Ill., USA; Forschungsinstitut Berghof GmbH., D-7400 Tübingen, p.o. box 1523

4.3.3 Low temperature wet ashing with .OH radicals from Fentons reagent H_2O_2/Fe(II)

In 1896, Fenton (39) observed, that organic substance can be strongly and unspecificly oxidized by boiling it in an aqueous solution of hydrogen peroxide in combination with small amounts of iron(II)sulfate. The astonishingly strong oxidising power compared to the mild conditions in a boiling dilute aqueous solution at temperatures below 100° 110°C could be explained only by assuming the action of radicals such as .OH or related ones, e.g. according to:

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + .OH.$$

Fenton's reagent was known in preparative organic chemistry at the turn of the century as an oxidative reagent for organic compounds, but it was too unspecific. In 1915, Mandel and Neuberg used this reagent for wet ashing of meat. Later this reagent was forgotten. In the laboratory of one of the authors in 1968 its powerful oxidation for wet ashing of different biological materials and foodstuffs has been rediscovered (96,) and used in routine work for several thousands of wet ashings in the last ten years (69, 70, 71, 72, 97, 98, 99, 100, 101). These include e.g.muscle, liver, kidney, whole blood, feces, urine; meat, whole fish; flower, starch, milk powder, rice, soya beans, peas, eggs, potatoes, bananas, water melon, spinach, salad, whole cabbage (1,8 kg in one open beaker operation!), tea, with sample weights between 100 g and 1.5 kg fresh weight.

The method is particularly suited for mild wet ashing of soft tissues such as muscle, organs (liver, kidney etc.), but is also easily applied to body fluids e.g. serum, whole blood, urine and whole animals such as fish, rat etc.

The method has been used mainly in two different types of operations, in an open beaker as well as in a closed system. In the open beaker operation, the minced or blended biological material such as soft tissue is boiled in 1 normal nitric acid medium for about one hour for hydrolysing the foaming substances and then evaporated with 30 % hydrogen peroxide to wet residue. This procedure is repeated after addition of small amounts of hydrogen peroxide, until a pure white ash is obtained. Ashing of 100 g meat from stag lasts about 4 to 5 hours, of 1 kg about 15 h; 300 g fish (whole macrel) about 8 h; 25 g dry blood 5 h; 500 g sugar 20 h; makerel 77 g feces 5 h; 160 g urine 5 h.

In the closed system, after the acid hydrolysis as described above 30 % hydrogen peroxide is added dropwise and slowly, to boiling aqueous suspension of the blended meat. Clear solutions are obtained after about 16 h.

In all the cases referred, fat is either not destroyed or only incompletely. But in most cases it can be easily removed, since it solidifies on cooling and can be mechanically separated after washing with hot 1 N HNO_3. Investigations on the trace element content of remaining fat showed that it carried no detectable amounts of lead and cadmium with it.

The general advantages of the method are: a)Large sample amounts up to the kilogram range and even more to allow a new general method for homogenizing soft tissues by ashing and dissolving large sample amounts, which are either a mixture of a large number of small samples or a whole organ such as liver or kidney. b) Due to the boiling aqueous solution it has the lowest ashing temperature of all ashing methods known (below 110°C). Therefore and because of the aqueous system, volatilisation of trace elements is minimum. So far only volatilisation of iodine (complete) and mercury (30-40 % losses) has been found. (c) Adaption to routine operation of up to ten or twenty samples; d) simplicity, reaction in dilute aqueous solution, under mild conditions, if necessary at neutral pH, e) open or closed system, f) short ashing times for complete ashing compared with usual muffle furnace dry ashing, especially for larger sample amounts, g) water and oxygen are the only reaction products of the reagent, h) no burden of acidic fumes, i) no excess of mineral acid, which has to be removed afterwards, k) almost no ash losses, l) 30 % hydrogen peroxide is commercially available (e.g. Peroxid Chemie GmbH, Hoellriegelskreuth near Munich, FRG or E. Merck Darmstadt). This reagent belongs to the purest commercialy available reagents and is purer than H_2SO_4, $HClO_4$, HF and even sometimes HNO_3. As for example, we found in 30 % hydrogen periode pro analysis from Merck Company only 0.03 Cd; 0.06 Co; 0.4 Cr; 0.1 Cu; 0.5 Fe; 0.5 Mn; 0.6 Ni; 0.1 Pb; 0.2 Zn (in ppb); m) the ash is water soluble.

A main disadvantage is incomplete or no ashing of fat, oil, coal and compact plastics. Others include: Addition of reagents and sometimes inactive carrier salts to avoid trace element losses by adsorption of the container walls, are a potential source of contamination. Nitrogen from proteins is kept in the solid residue or the ashed solution in form of ammonium ion. In most cases, this does not disturb subsequent steps. If necessary, the ammonium salt can be removed by sublimation. Sometimes foaming may occur during the initial stages but is easily eliminated by boiling the suspension sufficiently in 1 N HNO_3, as mentioned before.

4.4 Others

Some further ashing techniques shall be mentioned here, which are used until now only in a limited number of cases.

4.4.1 Oxidative fusion

Parr bomb, where oxidative fusion with Na_2O_2 has been used (9), is the most well known example of oxidative fusion. A microbomb has been reported (86) for small samples. Many other oxidants eg. potassium nitrate, potassium perchlorate have also been employed in conjunction with the oxide (17). Generally the process is carried out in a closed system but open systems have been used as well. Bowen (21) describes oxidative fusion with a mixture of sodium and potassium nitrates in an open borosilicate glass beaker, though doubts if the method could be of much use in determining inactive elements, because of the possibilities of contamination. The oxidative fusion of plant and biological material for estimating selenium has been described by Dye and co-workers (33).

4.4.2 Oxidation in nitric acid vapour

Gorsuch (46) refers to the possibility of destroying, organic matter by nitric acid vapour.

Thomas and Smythe (118) found vapour phase oxidation by nitric acid efficient for destroying up to 90 % organic material in 5 - 6 min, when addition of perchloric acid ensured speedy and complete oxidation. However, it would be doubtful if fat can be completely destroyed merely with the use of nitric acid vapour alone.

4.4.3 Oxidation with ozone

Oxidative decomposition of organic matter can be carried out with ozone (63), when the unsaturated groups are broken down to aldehydes and ketones. The mechanism may probably be through radical formation which is very effective.

4.4.4 *Reductive decompositions*

The reductive decomposition of organic matter with hydrogen has already been referred to. Various other reductants which have been used include (17), NaOH/NaCN (22), calcium hydride (24), stannous chloride (8), sodium formate (14), lithium alanate, (77, 112), ammonium dihydrogen phosphite (123), sodium borohydride (38). However, most of these have no importance in trace analysis because of the very high blank values.

4.4.5 *Decomposition with halogens*

The halogenation of organic substances has been used for decomposition and subsequent analysis for trace elements. Körbl, Mansfeldova and Vanickova (66, 67) decomposed organic materials in a stream of chlorine. The chlorination of organic matter in aqueous solution has been used for estimating mercury in urine (124). The use of bromine/hydobromic acid (30) and decomposition with bromine and concentrated sulphuric acid (40) have been reported. Alkyl and aryl mercury compounds are analysed for mercury by decomposition with iodine/hydroiodic acid (128).

4.4.6 *Enzymatic/Hydrolytic decompositions*

Enzymatic decomposition sometimes has been reported for destruction of organic matter eg. proteins. The use of enzymes eg. amylase (51) and amylolglucosidase (82) has been reported. Kracke et al. (68) report enzymatic decomposition of larger quantities of food samples for analysis of plutonium before wet ashing by H_2O_2/Fe^{2+}.

Sometimes radiation damage may also improve the ashing effect of other ashing methods.

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5. CONCLUSIONS

1. Unlike the basic sampling operation which may at times preclude the direct participation of the analyst, the entire responsibility during the sample preparation step rests on the analyst, who is the most important part of any analysis.

2. If the basic principles, outlined for obtaining of valid samples are observed during the sampling stage, with the help of prior knowledge of the nature and composition of the sample matrix, awareness of the cleanliness of one's own working environment and the limitations of the analytical technique to be used, greatly help to improve the accuracy of the results.

3. More details concerning sampling and sample handling procedures should be mentioned while reporting analytical procedures and results in order to help judging the validity of the data from sampling and sample preparation point of view.

4. There is an urgent need to critically evaluate some of the existing sampling and sample preparation procedures for various tissues in order to recommend some standard procedures and equipments. Only then it would make the intercomparisons and the utilization of the analytical results from various sources valid for wider applications such as nutritional and physiological studies covering various population groups.